PROTO-ONCOGENE EXPRESSION IN HUMAN CHONDROSARCOMA AND MALIGNANT FIBROUS HISTIOCYTOMA

By

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To my husband Ron, to my grandparents, to Karen and Ken, and to Mom and Dad; whose love, support, and belief in me will always be my inspiration.

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Total cellular RNA and genomic DNA were extracted from 20 chondrosarcomas, 23 malignant fibrous histiocytomas (MFH), 9 muscle, and 6 bone marrow specimens. Levels of RNA and gene copy numbers of c-myc, c-Ha-ras, c-fos, c-sis, v-erb-B-1, v-src, thymidine kinase (TK) and actin were quantified densitometrically from slot-blot analysis. C-myc, c-Ha-ras, and c-fos transcript levels are undetectable in muscle. Mean c-myc:TK ratios do not differ significantly among groups of bone marrows, chondrosarcomas and 17 MFHs ($p \geq 0.05$). Six MFHs have a mean c-myc:TK ratio of 2.0 which is significantly higher than the other groups ($p \geq 0.05$). Intergroup comparisons between bone marrows, chondrosarcomas, and

MFHs show no significant differences in expression of c-Ha-ras, c-fos, v-erb-B-1, and v-src. C-sis RNA levels are 2-to 3- fold greater in MFHs. DNA analysis shows **c-myc** to be a single copy gene in all tissues except 6 MFHs which have between 2 and 11 copies. **C-myc** amplicons were found to be large, extending at least 50 kb 5' to the **c-myc** promoter and slightly 3' of exon 3. These same tumors have increased levels of **c-myc** transcript as determined from RNA analysis. C-Ha-ras, c-fos, c-sis, **v-erb-B-1**, and **v-src** are single copy genes in all tissues.

Chromatin structure studies show that amplified **c-myc** in P3C cells does not contain a DNAse I hypersensitive site near the P0 promoter region 5' to exon 1, known to be involved in maintaining **c-myc** transcript production in HL-60 cells. Additionally, a new site is present in a region known to contain a block to transcription elongation in Burkitt lymphomas. These changes are not seen during normal upregulation of **c-myc** in G0 serum released fibroblasts (G0/G1 transition).

These data suggest that increased levels of c-myc expression are due to gene dosage, while those of **c-sis**

are due to some unknown mechanism other than gene amplification. Additionally, differences in chromatin structure between amplified and single copy c-myc in MFH cells may represent a compensatory response to increased c-myc transcript production. Increased levels of c-myc protein provide further evidence that c-myc may be an oncogene in these cells.

CHAPTER 1
INTRODUCTION

Mechanisms of Tumor Development

Fundamental requirements for successful prevention and sometimes treatment of cancer are knowledge and understanding of its causitive factors. This task is not an easy one by any means. Agents having abilities to contribute to or cause cancer are called carcinogens. Studies to determine the roles these agents play in neoplastic processes have focused on three general classes of carcinogens: chemical, physical, and biological.

Carcinogenic chemicals and ionizing radiation are known to affect DNA at a structural level and to be mutagenic under certain conditions. Therefore, one of the long-standing theories of carcinogenesis has been that cancer is caused by genetic mutations.

Evidence that chemicals can induce cancer has been reported for more than two centuries. The first observations of chemically induced cancer were made in humans. The first of these was in 1761 when Hill noticed that nasal cancer was

more common in people who frequently used snuff (117). In 1775, Pott reported a high incidence of scrotal cancer in men who were chimney sweeps (117). More discoveries of this nature were made in subsequent years, leading to attempts to induce cancer in animals with chemicals. One of the first succesful attempts was made in 1915 by Yamagiwa and Ichikawa who induced skin carcinomas by the repeated application of coal tar to the ears of rabbits (117). Subsequent studies focused on identifying the actual carcinogenic chemicals in the compounds which could induce cancer. Today, the list of known carcinogenic chemicals is quite extensive and includes a wide variety of different chemicals. Examples of carcinogenic substances include industrial chemicals such as aromatic hydrocarbons, halogenated hydrocarbons, nitrosamines, intercalating agents, alkylating agents, nickel and chromium compounds, asbestos, vinyl chloride, diethylstilbesterol, and certain naturally occurring substances such as aflatoxins and radon gas.

Chemical carcinogens are capable of interacting with a wide variety of cellular macromolecules. This usually involves the alkylation of nucleophilic groups on nucleic acids or the reaction of electrophilic groups of the carcinogen with proteins (142). Some chemical carcinogens

are known to react with cellular RNA as in the case of dimethylnitrosamine (105), but most react with DNA (142). Reaction of chemical carcinogens with DNA can facilitate the induction of heritable changes in cells and may lead to malignant transformation. Thus, it is generally believed that this is the most likely mechanism for chemical initiation of carcinogenesis. Representative agents from virtually all classes of chemical carcinogens have been shown to affect DNA in some way. The actions of many of these have been found to result in the formation of base-adducts. The potential biological consequences of these are are several: Base adducts may stabilize intercalation reactions. For example, if the flat planar rings of a polycyclic hydrocarbon were stably integrated between the stacked bases of double helical DNA, the helix would be distorted. This could lead to a frame-shift mutation which would occur during DNA replication past the point of intercalation (86).

Many of the base adducts formed by carcinogens involve modification of N-3 or N-7 positions on purines. This induces an instability in the glycosidic bond between the purine base and deoxyribose. The destabilized structure can then undergo cleavage by DNA glycosylase, resulting in loss of the base, and creation of an apurinic site in the DNA.

This open space can then be filled by any base, resulting in a base transition (purine-pyrimidine base change) (110).

Interaction with some carcinogens has been shown to favor a conformational transition of DNA from its usual double-helical B form to a Z DNA form (125). This could alter the ability of certain genes to be transcribed, since B-Z conformational transitions are thought to be involved in regulating chromatin structure (142).

Both X-rays and ultraviolet radiation also produce damage to DNA. As with chemical carcinogens, this damage induces DNA repair processes, some of which are error prone and lead to mutations. Studies have shown that the development of malignant transformations in cultured cells after irradiation requires fixation of the initial damamge into a heritable change. This is experimentally accomplished by allowing clonal proliferation and expression of the transformed phenotypes (109).

In addition to chemical and physical carcinogens, biological carcinogens exist as well. It was long suspected that various forms of cancer, particularly certain lymphomas and leukemias, were caused or at least cocaused by transmissable viruses. The known carcinogenic effects of certain chemicals, irradiation, chronic irritation and

hormones did not fit with the notion of an infectious origin of cancer. Early studies attempted to transmit malignant disease by inoculation of filtered extracts prepared from diseased tissues. It was later demonstrated by Ellerman and Bang in 1908 that chicken leukemia could be transmitted by cell-free filtered extracts (50). They were among the first to demonstrate the viral etiology of this disease. An example of a virus thought to cause cancer in humans is seen with Human T-cell leukemia virus 1 (HTLV-1), a transmissible virus thought to cause leukemia (133).

Other oncogenic RNA viruses are capable of participating in transformation. Understanding of the molecular mechanisms involved in cellular transformation by these viruses is based on the Nobel prize winning work of Baltimore and Temin (5, 173). In the early 1960s, Temin demonstrated that mutations in the Rous sarcoma virus (RSV) genome of RSV-infected chicken cells could be induced at a high rate. It was also shown that mutation of an RSV gene present in an infected cell often changed the morphology of the cell, and the virus genome was stably inherited by progeny cells (173). This led to the notion that virus genetic information was contained in a regularly inherited structure of the host cell as a "provirus" integrated into the host cell's genome. The

problem with the provirus hypothesis was that there was no known way for the tumor virus RNA to be converted into DNA and integrated. Temin and Baltimore independently demonstrated the presence of a virus coded, RNA directed, DNA polymerase activity now known as reverse transcriptase (4, 174). As a result of this work, Temin proposed the "protovirus" theory in which he postulated that genomes of oncogenic viruses arose during evolution from normal cellular DNA altered by some exogeneous carcinogen (173).

The normal cellular homologues of viral oncogenes (v-onc) are known as proto-oncogenes (c-onc). These are thought to have been evolutionarily conserved in the genomes of most animal cells over a long period of time. They seem to be involved in control of cellular growth and proliferation. It is likely that their activations to oncogenic states occur from one or more rare events such as translocations, amplifications, point mutations or other aberrations of key nucleotide sequences (102). Highly oncogenic viruses presumably arose from genetic recombination events between viruses of low oncogenicity and proto-oncogenes. The combination of these two elements seems to have produced highly transforming viral genomes. Many of these viruses are replication defective, and do not form complete viruses

unless coinfected with a "helper" virus. Recombination between replication-competent helper viruses and cellular genes also may have produced highly oncogenic virus strains.

The "oncogene" hypothesis of Huebner and Todaro (88) postulates that the cells of most or all vertebrates contain "virogenes". These genes include sequences responsible for transformation and are transmitted vertically form parent to offspring. In this hypothesis, the occurrence of cancer may be determined by the derepression of endogenous viral oncogenes. Activation of repressed genes could result from exposure of cells to chemical carcinogens, irradiation, normal aging, or a combination thereof. This theory provides an explanation for the known vertical transmission of certain animal viruses. It also explains the observed necessity of synergistic interactions between chemical carcinogens and irradiation for transformation by some oncogenic viruses.

Multistep Carcinogenesis

The idea that development of cancer is a multistage process arose from early studies of virus induced tumors, and from discovery of cocarcinogenic effects of croton oil. Rous discovered that certain virus induced skin papillomas in

rabbits regressed after a period of time. The papillomas could be made to reappear if the skin was stressed by punching holes in it or treating it with irritants such as turpentine or chloroform (142). These experiments led to conclusions that tumor cells could exist in a latent or dormant state, and tumor induction processes and subsequent growth of the tumor involved different mechanisms. These mechanisms are known as initiation and promotion (62).

Studies of events involved in the initiation and promotion phases of carcinogenesis were greatly aided by isolation and identification of initiating agents such as urethane, and the purification of the components of croton oil which had promoting activities. The promoting substances were found to be diesters of the ditepene alcohol; phorbol (84). Of these, 12-O-tetradecanoylphorbol-13-acetate (TPA) is the most potent promoter (11).

Initiation of transformation in normal cells by a carcinogenic agent involves a permanent, heritable change in gene expression. This could occur by direct genotoxic or mutational events, where the carcinogenic agent reacts with DNA directly. It may also occur via indirect "epigenetic" events which regulate gene expression without direct interaction with DNA sequences. Many feel initiating events

have a direct impact on DNA itself. According to Ruddon (142), this theory depends on three kinds of evidence.

1. Agents which damage DNA are frequently carcinogenic. It has been shown that chemical carcinogens are usually activated to generate electrophilic agents. These form specific reaction products with DNA. In some cases, as with alkyl O-6-guanine, the extent of product formation has been shown to correlate with mutagenicity and carcinogenicity of the agent (142).

2. Most carcinogenic agents are mutagens. A number of *in vitro* test systems using mutational events in microorganisms have been developed to rapidly screen the mutagenic potential of various chemical agents. One of the best known is the Ames test. Ames and his collegues have shown that about 90 percent of all carcinogens are also mutagenic (114). Very few noncarcinogens showed significant mutagenicity in this test system.

3. The incidence of cancer in patients with DNA-repair deficiencies is increased. In individuals with certain recessively inherited disorders, the prevalence of cancer is significantly higher than in the general population (103, 153). The common characteristic shared between these disorders is the inability to repair some kinds of physical

or chemical damage to DNA. Such examples include, xeroderma pigmentosum (deficiency in excision repair), ataxia telangiectasia (greater sensitivity to X-irradiation, more prone to leukemia and other cancers), Fanconi's syndrome (deficiency in repair of cross-linked bases, repair of X-ray or UV induced damage), and Bloom's syndrome (increased propensity to develop cancer, high genetic instability of chromosomes). The high incidence of cancer in patients with these diseases constitutes the best available evidence for a causal relationship between mutagenicity and carcinogenicity in humans (168, 169, 185).

Tumor initiating agents most likely interact with DNA to induce mutations, rearrangements or amplifications, producing a genotypically altered cell. The initiated cell then undergoes clonal expansion influenced by promoting agents which act as mitogens for the transformed cell (142). It has been suggested that promoting activities may be mediated by cellular membrane events. Direct action of promoters on DNA has also been proposed (142). As a result, multiple clones of cells are likely to be initiated by a DNA damaging agent. Then, through a rare second event, one or a small number of these clones progresses to malignant cancer.

Tumor promotion is itself a multistage process sometimes labeled collectively as "tumor progression". Tumor promotion is thought to be a stage of cell proliferation and clonal expansion induced by mitogenic stimuli. The progression phase is the evolution of genotypically and phenotypically altered cells resulting from genetic instability (128). During tumor progression which can take years in humans, individual tumors develop heterogeneity with respect to their invasive and metastatic characteristics, antigenic specificity, state of differentiation, and response to drugs and hormones (128). It is thought that some major selection process occurs to favor the growth of one cell over another, thus a dominant clonal population of cells may emerge. This may be a result of competition for nutrients, ability to evade the immune system, and resistance to chemotherapeutic drugs.

The concepts of initiation and promotion support the notion that cancer is not a "one-hit" event. Evidence obtained from studies done with oncogenes and antioncogenes further supports this concept. Weinberg (102) showed that when rat endothelial fibroblasts were transfected with the c-Ha-ras and c-myc oncogenes alone, no transforming effect was observed. However, when c-**myc** and c-**Ha-ras** were

transfected together, multiple foci of transformed cells were obtained. These cells had the capabilities to grow very rapidly in culture and seed tumors in nude mice. Acting together, c-myc and c-Ha-ras could do what neither gene could do on its own.

Additional evidence for multistep carcinogenesis is seen with the retinoblastoma (rb) gene. Retinoblastoma is a childhood ocular tumor which requires both alleles of the rb gene to be mutated in order for the disease to occur. Knudson (97) has proposed that the rb gene behaves as an "anti-oncogene", in that one normal allele is sufficient to protect against the disease. His "two hit" model for this disease suggests that two mutagenic events occur at 13q14 of chromosome 13. These two events can be in the form of 2 germline events, 1 germline and 1 somatic, or two somatic. The rb gene is now thought to have an involvement in other human malignancies including osteosarcoma (79) and mammary carcinoma (56,80), and its activation provides an example of multistep cancer in humans.

The Neoplastic Phenotype and Steps of Tumor Progression

Much effort has gone into comparing phenotypic characteristics of *in vitro* transformed cells with those of

cancer *in vivo*. These types of studies have greatly increased understanding of cancer cell biochemistry. Unfortunately, many biochemical characteristics of cultured cells are dissociable from their abilities to produce tumors in animals (142). Furthermore, individual cells of malignant tumors from animals and humans exhibit extensive biochemical differences. These differences are reflected in cell surface composition, enzyme levels, immunogenicity, and response to cancer drugs.

Some general characteristics of transformed malignant cells growing in culture include the following (142):

1. Histiologic characteristics of malignant cells *in vivo*. The nuclei are increased in both size and number, and there is a great deal of variation in the sizes and and shapes of the cells. Also, there are increased nuclear:cytoplasmic ratios, and the formation of clusters of cells may be observed.
2. Differences in growth characteristics are common:
   a. Transformed cells in culture are immortalized. Malignant transformed cells can be passaged in culture for an indefinite period of time.
   b. Transformed cells tend to pile up in culture and are not subject to contact inhibition seen with

normal cells. As a result, malignant cells in culture may grow to a much greater density.

c. Transformed cells seem to have much lower requirements for serum and/or growth factors to survive in culture than normal cells do.

d. There also seems to be a loss of anchorage dependence with transformed cells. They may no longer need to grow attached to solid surfaces, and can grow in soft agar.

e. It has been observed that when transformed cells in culture are subjected to biochemical restrictions, they do not stop growing. An example of this is a lack of response to serum starvation.

3. In vitro transformed cells may also change their surface properties. Changes of this nature include; alteration in structure of surface glycolipids and glycoproteins, loss of surface fibronectins, increased agglutination by lectins, changes in surface antigens which may be tumor specifc and involved in immune responses, and increases in the degree of amino acid uptake.

4. Cultured malignant cells produce increased levels of the enzymes involved in DNA synthesis. They also

produce higher levels of other enzymes such as proteases and collagenases.

5. Different transformed cells have varying levels of nucleotides. Some may have higher cAMP levels or increased cGMP:cAMP ratios than their normal cell counterparts.
6. Transformed cells in culture have been shown to produce growth factors involved in tumor growth. These include angiogenesis factors, and transforming growth factors (TGF). These may be produced to favor their own growth (autocrine function).
7. Fetal antigens, placental hormones, and fetal enzymes have been shown to be produced in increased amounts in cultured tumor cells. This is characteristic of tumor cells in vivo.
8. Ability to produce tumors in experimental animals is a characteristic of malignant cells.

In addition to biological and physiological changes in transformed cells, changes at the molecular level occur as well. Genetic instability during tumor progression is characterized by a variety of aberrations in the genome including point mutations, deletions, rearrangements, amplifications, chromosome translocations and abnormal

chromosome number (aneuploidy). It is thought that aberrations such as point mutations, deletions, and rearrangements are events associated with initiation processes, whereas gross chromosomal changes occur as the tumor progresses in malignancy (142). There are certain chromosomal deletions, translocations and trisomies which are characteristically associated with specific cancers. These are called non-random chromosomal alterations. Changes in ploidy are associated with many tumor types in advanced stages, and are somewhat random in that no definitive pattern of chromosome number is associated with a particular tumor type. In more advanced cancers both random and non-random chromosomal changes can be found. Continuous chromosomal changes can bring about tumor heterogeneity and the selection of more highly invasive and metastatic cancers. Thus, tumor progression has been called a highly accelerated evolutionary process.

Malignant tumors have several important in vivo characteristics. At the cellular level, they have a greater fraction of cells in S-phase, and are less differentiated than their normal counterpart tissues. In order for tumor cells to grow, divide, and metastasize, cell growth must outnumber cell death. Therefore, angiogenesis factors are

important to the growth of malignant tumors, as rapidly growing tumors often outgrow their blood supplies. It is thought that malignant tumor cells may produce their own growth factors, angiogenesis factors, and collagenases, enableing them to compete with other cells for nutrients, and eventually invade surrounding tissues.

Specific Questions Addressed During the Course of This Project

Unrestrained cell growth is a common component of neoplastic phenotypes. Proto-oncogenes are genes which have been shown to be involved in regulation of cellular growth and differentiation. They are found in all normal nucleated animal cells. Their conversion to transforming genes or oncogenes by one or more of several possible mechanisms may allow the transformation of cells *in vitro* and generate neoplasms *in vivo*. Exploration of how these potential regulators of growth control interact with one another and with other genomic components may enlighten our understanding of how normal cellular replication or differentiation events change with transformation.

It is possible that several proposed mechanisms of proto-oncogene activation will lead to increased production

of transcript. Examination of gene copy numbers and gene expression will offer clues to possible mechanisms involved in activation of proto-oncogenes. The following was a general basis for this project:

> Normal cellular genes, when mutated by several suggested mechanisms, may contribute to the tumorigenesis and biologic behavior of chondrosarcoma and malignant fibrous histiocytoma.

From this, the following hypotheses were derived:

1) Increases in proto-oncogene transcript levels may be due to gene amplification.
2) There are differences in chromatin structure between amplified and single copy proto-oncogenes.

To test the first hypothesis, tumor RNA and DNA samples were evaluated for proto-oncogene transcript levels and gene copy numbers of **c-myc**, **c-Ha-ras**, **c-fos**, **c-sis**, **v-erb-B-1**, and **v-src**. These genes were studied because of previous associations with sarcomas in humans and other animals.

It was desirable to study potential regulatory changes which accompanied proto-oncogene amplification and increased transcript production. Therefore, the second hypothesis was tested by studying the locations of DNAse I hypersensitive sites. These sites represent areas where regulatory interactions are thought to occur. Changes in locations of these

sites may offer clues to regulatory mechanisms involved in proto-oncogene transcript production.

CHAPTER 2
REVIEW OF THE LITERATURE

Differentiation of Mesenchyme

The precise pathways taken by mesenchymal cells undergoing differentiation have been somewhat of a controversial issue. Therefore, two proposed models of mesenchymal differentiation will be presented here. The first of these models, the radial model of mesenchymal differentiation, is a currently accepted model proposed by Hadju (76). Each soft tissue and hematopoietic phenotype directly originates from a primitive undifferentiated mesenchymal cell. In this scheme, there are no precursor cells and no branching of cell types. Rather, the differentiated phenotypes are separated from each other only by a primitive mesenchymal cell. Because of this, close relationships exist between phenotypes.

More recently, another model has been proposed by Brooks (23) and is shown in figure 1. The two novel features of this model are the insertion of an intermediate precursor

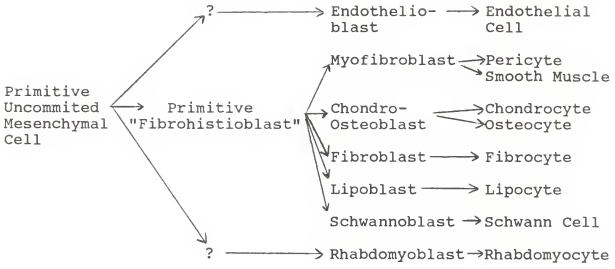


Figure 1. Hypothetical model of mesenchymal differentiation as proposed by Brooks. *

* Taken from Brooks, J.J. 1986. The Significance of Double Phenotypic Patterns and Markers in Human Sarcomas. A New Model of Mesenchymal Differentiation. Am. J. Pathol. 125: 113-123.

between the primitive cell and some differentiated phenotypes, and a branching system reflecting close relationships between some phenotypes and not others. This model also recognizes the myofibroblast and the chondroosteoblast.

Chondrosarcoma and Malignant Fibrous Histiocytoma

Chondrosarcoma is a malignant tumor of cartilage (figure 2). It has been well established that the basic proliferating tissue is cartilagenous (39). Primary chondrosarcomas are tumors which can arise de novo in extraskeletal tissues or in mixed tumors such as teratomas. The majority of chondrosarcomas are "myxoid". Those composed of hyaline cartilage are more uncommon. Secondary chondrosarcomas arise most commonly in osteosarcomas, and can sometimes develop in patients with multiple exostoses. Rarely do they develop from an enchondroma, a benign cartilagenous tumor. In addition to primary and secondary chondrosarcomas there are dedifferentiated chondrosarcomas which give rise to more malignant tumors such as osteosarcomas, fibrosarcomas, or malignant fibrous histiocytomas (MFH) (39).

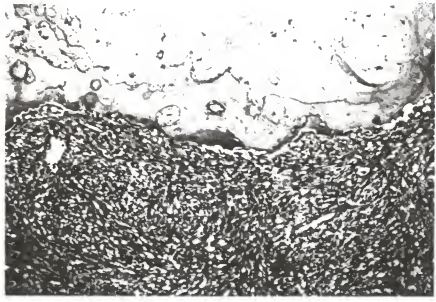


Figure 2. Histiologic appearance of chondrosarcoma. This section was taken from a patient with areas of grade I (less dense cellularity) and grade III tumor. The appearance of the grade III area closely resembles that of an MFH (shown below).

Chondrosarcoma is primarily a tumor of adulthood (39). The incidence of bone tumors in general is highest during adolescence with a rate of 3 per 100,000 (61). The incidence falls to 0.2 per 100,000 at ages 30-35 and rises slowly thereafter to an incidence rate equal to that of adolescence (30, 39). Chondrosarcoma is the third most common type of bone tumor and makes up approximately 13 percent of all malignant bone tumors (85). More than 75 percent of chondrosarcomas occur in the trunk and the upper ends of the femora and humeri. It is much less common for these tumors to be located in the distal extremeties such as the elbows and ankles (39).

Many chondrosarcomas are palpable, but many of those affecting the trunk or long bones of the extremeties which have not broken the cortex may cause pain alone to indicate the presence of the lesion. Roentgenograms provide a very helpful means for diagnosis. Osseous destruction in the area of the lesion combined with irregular densities from calcification and ossification are commonly observed. Central chondrosarcomas of long bones commonly produce fusiform expansion of the shaft associated with thickening of the cortex (39).

Chondrosarcomas usually have a slow clinical evolution. Metastasis is relatively rare and occurs late. The basic therapeutic goal is to control the lesion locally and to prevent local recurrence. Therefore, radical early surgical treatment is desirable (39). A long followup after treatment is necessary because recurrence may develop many years later. The overall survival is approximately 50 percent at 5 years (180).

With respect to prognosis, the correlation between poor differentiation, rapid growth rate, and metastasis is high. Clinical study results suggest that a high cure rate is expected for patients with more differentiated tumors (135). A grading system exists for chondrosarcoma and is important in terms of predicting survival and establishing the most effective treatment protocol (39).

Criteria for grading chondrosarcomas are those of Evans et al. (57), and include the following: Grade I tumors have the presence of or domination of cells with small densely staining nuclei, an inter-cellular background of a chondroid or myxoid nature, frequent calcification patches, and multiple nuclei present within a single lacuna. Grade II tumor characteristics include; areas where a significant fraction of the nuclei are of a moderate size, a mitotic

index of 0-2 per 10 high power fields (40X), dense cellularity, paler staining nuclei, a background which is more more myxoid than chondroid, and a greater cellularity /increased nuclear size limited to isolated areas.

The criteria for the classification of a chondrosarcoma as grade III are a mitotic index greater than 2 mitoses per 10 high power fields (40X), increased nuclear size compared to those of grade II tumors, very dense cellularity which may appear MFH like, and the absence of a chondroid or myxoid background.

Malignant fibrous histiocytomas (MFH) (figure 3) are soft tissue tumors whose cell of origin has been disputed, but current evidence indicates that these are are immature mesenchymal cells (15, 90). Malignant fibrous histiocytomas usually occur in deeper structures such as deep fascia and skeletal muscle. They also have been seen in soft tissues of the extremities, mediastinum, and retroperitoneum, and may occur within bones in areas of infarction or prior radiation. As a group, these tumors comprise only about 0.8 percent of all bone tumors (39), but are somewhat more common in soft tissues. There seems to be a slightly higher percentage of males with this disease than females, and nearly any age may be affected (190). As with other varieties of bone tumors,

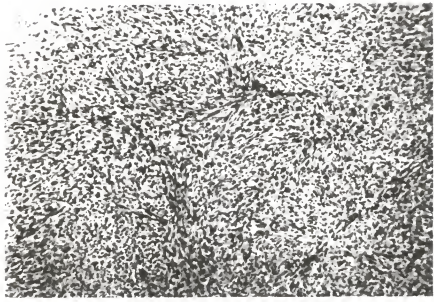


Figure 3. Histiologic appearance of MFH.

pain and swelling are the most frequent symptoms. As with chondrosarcomas, roentgenographic analysis is helpful in preliminary diagnosis of the tumor.

Histiologic features of these tumors usually include a high degree of variation, multinucleated tumor cells, nuclear hyperchromasia and a high mitotic activity. A typical pattern of growth can be described as the arrangement of tumor cells around a central point, producing radiating spokes, grouped at right angles to each other (storifiorm pattern).

With respect to prognosis, subcutaneous tumors generally have a better prognosis than the more deep seated lesions. The recurrence of MFHs is said to be 44 percent with a two year survival of 60 percent (190). There is no widely accepted grading system for deep seated MFHs as for chondrosarcomas. Other important prognostic factors include mitotic index, degree of cellular polymorphism, tumor size and tumor stage (180).

Staging criteria for both chondrosarcomas and MFHs are those described by Enneking et al. (52) for sarcomas originating from mesenchymal tissue of the musculoskeletal system. This system takes into account surgical grade, local extent, and presence or absence of regional

metastasis. Grade is further classified as low and high, local extent as intracompartmental and extracompartmental, and the extent of regional or distant metastasis is defined as either present or absent.

Proto-Oncogenes

During the early 1970s it was discovered that a single gene carried by a retrovirus could cause cancer in animals. Soon thereafter, it was thought that the oncogenes acquired by retroviruses might be derived from normal cellular genes present in the host. It was later shown by Stehlin et al. (164) that cDNA specific for the v-src region of Rous Sarcoma Virus could detect closely related sequences in the genome of normal chicken cells. This gene, now called c-src has been found in all other vertibrate species including man (162).

Since the discovery of cellular sequences homologous to v-src, cellular counterparts (c-onc) for the other viral (v-onc) oncogenes have also been found (16). These cellular sequences are known as cellular oncogenes or proto-oncognes. There are now more than forty known proto-oncogenes which are expressed in most normal mammalian cells (77). The c-ras and

c-myc genes are transcribed in almost all mammalian cells (levels may be low at about 5-20 molecules of RNA per cell) whereas most other proto-oncogenes seem to be more tissue specific (77). For example, **c-myb** is expressed in hematopoietic cells but not elsewhere (191). **C-sis** RNA has been detected in very few normal cell types, including rapidly dividing cells of the human placenta and endothelial cells (8). Since proto-oncogenes are so conserved between species, it seems likely that their gene products play an essential role in normal cellular growth and development.

There are several possible mechanisms by which proto-oncogenes may be activated to oncogenes. The first of these mechanisms involves insertional mutagenesis. The over expression of a proto-oncogene may occur after the integration of a new promoter. For instance, the **c-mos** proto-oncogene of mice which is biologically inactive after molecular cloning, can be experimentally converted into a potent oncogene by additon of a strong transcriptional promoter (18). Another example of this mechanism comes from similar activation of the c-Ha-ras proto-oncogene of rats (40). These oncogenes are created by ligation of cloned DNA segments, and acquire transforming capabilities because their transcripts are produced at much higher levels than

those afforded by native promoters of the normal proto-oncogenes. In vivo, the c-myc and c-erb-B-1 proto-oncogenes present in several avian hematopoietic neoplasias have become activated after adjacent integration of an avian leukosis proviral DNA segment. This viral segment provides a strong transcriptional promoter which replaces indigenous promoters of these genes (83, 131).

A second mechanism of activation involves overexpression due to amplification of the proto-oncogene (gene dosage effects). The c-myc proto-oncogene is amplified 30-50 times in HL-60 promyelocytic leukemia cells (32), and in a neuroendocrinal tumor the the colon (1). A c-Ki-ras gene is amplified 3-5 times in a human colon carcinoma cell line (115), and 60 fold in an adrenocortical tumor of mice (149). Human neuroblastomas were found to contain 30-100 copies of the N-myc gene (150). This was later confirmed, and shown to be associated with patient survival (152). A human chronic myelogenous leukemia cell line was discovered to have multiple copies of the c-abl gene (33). In each of these cases, gene dosage effects are thought to be responsible for increases in transcript levels and gene product.

A third mechanism involves enhancer/promoter activity. Enhancer sequences may increase utilization of transcriptional promoters to which they become linked. The affected promoter may be several kilobases away in either 5' or 3' directions (74). One example of this is the presence of retrovirus genome fragments downstream from the c-myc gene in some avian lymphomas (131). Here, the retrovirus elements appear to act by contributing an enhancer sequence rather than a promoter. It is entirely possible that point mutations at key regulatory sites such as promoter regions rather than coding regions may result in in proto-oncogene activation. This could facilitate the deregulation of a proto-oncogene, i.e. one with abnormal transcriptional control, or one which is inappropriately expressed.

A fourth mechanism involves the **c-myc** gene in particular. Work with Burkitt lymphomas has demonstrated the juxtaposition of the **c-myc** gene and immunoglobulin genes following a translocation event. As a result of this translocation, the **c-myc** gene loses all or part of its own regulatory exon and acquires normally unlinked sequences involved in immunoglobulin production (104). Rearranged **c-myb** sequences have been found in certain mouse plasmacytomas

(122) but their detailed structure and mechanism of activiation remain to be elucidated.

The fifth mechanism centers around structural alterations in the proto-oncogene and protein product. This mechanism is well documented in the case of the oncogenic proteins encoded by the **ras** genes. It was discovered that in the case of the oncogene in the T24/EJ human bladder carcinoma cell line, a point mutation at position 12 converted the c-Ha-**ras** proto-oncogene to an oncogene. This G to T transversion causes glycine which is normally the 12th residue of the encoded 21,000 dalton protein to be replaced by a valine (170). Another activated version of this gene encodes an aspartate residue at this position (144). Studies done with genes of the **Ki-ras** group also showed that when the 12th residue was altered in this manner, oncogenic activation of the **c-Ki-ras** gene was observed (24). A slight variation of these results was obtained through the study of a human lung carcinoma **c-Ha-ras** oncogene found to have a mutation at amino acid 61 of the p21 protein (198). These changes do not seem to affect the levels of expression of these genes, only the activities of encoded proteins. It is therefore suggested that the codons specifying residues 12 and 61 represent critical sites which, when mutated, will

often generate oncogenic alleles. It seems that point mutations elsewhere in the **ras** proto-oncogenes merely serve to inactivate the genes instead of converting them to oncogenes (102).

Finally, the possibility that unknown mechanisms of activation may be at work must not be overlooked. There may be mechanisms of activation which have not been determined. It is possible that new mechanisms may eventually be implicated in proto-oncogene activation.

Biochemistry of Oncogene Products

Cytoplasmic Kinases

One of the first oncogene proteins of this class to be recognized and studied was the 60,000 dalton protein of the **v-src** gene (pp60 **v-src**) (89). Other oncogene products with tyrosine-specific protein kinase activity include **yes**, **abl**, **fps**, **fgr**, and ros (6, 77). These proteins are all located at the inner surface of the cytoplasmic membrane and a comparison of their amino acid sequences has shown that they are related to each other (35). A region of approximately 250 amino acids in pp60 **src** is responsible for

the kinase activity, and a corresponding domain is found in other tyrosine kinases with a high degree of amino acid conservation between them. This kinase domain is also found in the cytoplasmic cyclic AMP dependent serine protein kinases in mos and raf, and in serine specific kinases located in the cytosol (118). A similar sequence domain has been found in the membrane-bound receptor-like products erb-B-1, fms, and neu, all of which have tyrosine kinase activity, indicating a distant evoloutionary relationship between all protein kinases (77).

Originally, it was thought that this activity was exclusive to oncogenes. However, a protein derived from the c-src gene was isolated from normal cells and shown to have tyrosine specific kinase activity (31). Since then, other membrane-bound cellular proteins with similar activities have been identified. These have offered clues as to what the oncogene kinases may be doing. It has been shown that the receptors for platelet derived growth factor (PDGF) and insulin-like growth factor (IGF) have a tyrosine-specific kinase activity (77). It has also been proposed that tyrosine phosphorylation is an early event in the transduction of mitogenic signals through the membrane. Although pp60src resides at the inner surface of the

membrane, and does not posess receptor activity, it probably does play some role in the early signalling process (35). Therefore, the presence of a tyrosine kinase encoded by a viral oncogene might result in a continuous, deregulated mitogenic signal for cell division.

Just how the cell responds to these signals is presently unknown. Many attempts have been made to find the cellular targets for phosphorylation by pp60**src** and by growth factor receptors. One effect of pp60**src** which is thought to be of importance is that it leads to increased protein phosphorylation on serine residues (38). The phosphorylation of the S6 ribosomal protein on a serine residue is thought to be a critical event in the mitogenic stimulation of normal quiescent cells. This may occur via a serine kinase intermediate which might be activated directly or indirectly by the pp60src tyrosine kinase (38).

Two different biochemical pathways have been shown to be important in the mitogenic stimulation of cells and a possible involvement with both has been shown for pp60**src**. Both of these pathways involve the generation of second messengers. The first involves the generation of cyclic AMP by membrane bound adenylate cyclase, leading to increased

levels of intracellular cyclic AMP. This can lead to the activation of cytoplasmic cyclic AMP-dependent serine specific protein kinases. Activation of other serine specific kinases, particularly protein kinase C may then occur (59). It is protein kinase C which is thought to play a central role in the various responses to mitogenic stimulation. Graziani (66) has shown that tyrosine phosphorylation of the cyclic AMP dependent protein kinases by pp60 c-src occurs in transformed cells. Therefore, it is possible that pp60src interacts with the pathway which regulates cell proliferation through cyclic AMP and protein kinase C.

Another pathway in which src may be involved also leads to the activation of protein kinase C. Full activity of this protein requires two cofactors; calcium, and diacylglycerol (127). Both of these can be generated in response to extracellualr signals such as acetylcholine or PDGF. The result of the interaction of these molecules with their receptors is a breakdown of inositol phospholipids located in the membrane to yield diacylglycerol. This activates protein kinase C, and inositoltriphosphate which can affect calcium levels within the cell. Sugimoto et al. (167) showed that pp60src could phosphorylate inositol phospholipids <u>in vitro</u>

and that in RSV transformed cells there is a buildup of intermediates in the inositol lipid breakdown pathway. They postulated that the primary target of pp60**src** might be lipid and not protein.

Much remains unknown about the biochemical action of pp60**src** and the rest of the tyrosine kinase family. Phosphorylation of tyrosine seems to be a general phenomenon for initiating cell division and inappropriate tyrosine kinase activity could explain the loss of growth control associated with transformed cells. The phosphorylation of inositol lipids by at least two of the tyrosine kinases **src** and **ros** is interesting, but the significance in transformed cells remains to be determined.

Ras Proteins

The 21,000 dalton (p21) proteins of three human cellular **ras** genes; Harvey (**Ha**), Kirsten (**Ki**), and **N-ras** are very closely related in sequence. In the first 150 amino acids there are a maximum of 14 amino acid differences between the three proteins. The **ras** proteins therefore have been highly conserved throughout evolution, and are thought to play an essential role in cell growth (77). The **ras** p21s are

located at the inner surface of the plasma membrane and although the viral proteins are phosphorylated at amino acid residue 59 which is a threonine, the human p21s do not have a threonine at 59, nor are they phosphorylated. The ras genes, which are cell cycle dependent (94), are activated by point mutations and therefore the modes of action of normal and transforming p21s are of interest.

Both transforming and normal cellular p21s bind GTP and GDP equally and have a GTPase activity (116). However, the transforming version of p21 hydrolyzes GTP about 10 times more slowly than the normal proteins (77). The normal ras roteins are thought to interact with a receptor in response to an external signal, bind GTP and interact with an as yet unknown molecule to generate a second messenger (77). Adenylate cyclase is unlikely to be directly involved because the G proteins associated with it have different molecular weights from ras p21 (77). Since transforming p21 has reduced GTPase activity, this could result in abnormally high levels of the second messenger.

Ras encoded proteins are also regulators of inositol triphosphate. Some of the proteins involved in the inositol lipid breakdown pathway are GTP binding proteins and it is possible ras may be one of these. Calcium has long been

implicated in cell proliferation. The increase in intracellular calcium which occurs when cells are fertilized or stimulated by growth factors may depend on the formation of inositol triphosphate. This can act as a second messenger to release intracellular stores of calcium. It has been postulated, and some evidence exists that the activated ras gene protein which binds but cannot hydrolyze GTP, can initiate the formation of inositol triphosphate in an uncontrolled fashion, independant of cellular growth factors (12).

In order to expand the current understanding of the functions of ras proteins, it will be necessary to identify which protein(s) they interact with in the cell. Attempts which have been made to copreciptiate ras associated proteins with anti-ras antibodies have been unsuccessful, indicating either that associations are weak, or they depend on intact membrane structure (77).

Growth Factors and Their Receptors

Certain oncogene products are known to be transforming versions of a growth factor and several growth factor receptors. The erb-B-1 oncogene is a truncated version of the

epidermal growth factor (EGF) receptor gene (46, 82). The neu (erb-B-2) oncogene, first detected by transfection assays has homology with erb-B-1 and also encodes a receptor-like molecule (145). The **sis** oncogene codes for one subunit of PDGF (45). Recently, it has been shown that **v-fms** is derived from the normal cellular gene encoding the receptor for colony stimulating factor 1 (CSF-1) (111, 146).

The erb-B-1 oncogene protein is different from the normal EGF receptor in that the extracellular EGF binding domain is absent (17). It is possible that this truncated receptor is in an activated configuration even in the absence of EGF stimulation. More about the erb-B-1 oncogene product will be discussed below. It has been predicted that other known growth factor receptors in addition to those for EGF and CSF-1 such as those for PDGF could be altered or inappropriately expressed to yield oncogenic proteins. So far no spontaneous examples of this have been reported.

Oncogenic changes in a growth factor protein are well exemplified in the case of the **sis** oncogene product. The c-sis protein sequences are homologous to one of the chains of PDGF, and are normally produced in only a restricted number of cell types; including bone marrow megakaryocytes (77), human placental cells, and endothelial cells (8). Receptors

for PDGF have been found mainly on mesenchymal and glial cells (165). In the case of virally transformed fibroblasts, the v-sis sequences are fused to the env sequences of the virus and this allows export of the abnormal PDGF-like molecule to the membrane.

Abnormal expression of any mitogenic factor such as sis may make it a possible candidate for a role in oncogenesis, providing the cells which produce it have the appropriate receptors. It is thought that high levels of sis expression cause transformation, presumably by autocrine stimulation via the PDGF receptor. The sis oncogene protein will be discussed in further detail below.

Many tumor cells release transforming growth factors (TGF). One class of these, TGF alpha is closely related in sequence to EGF and interacts with the EGF receptor (43). Other evidence suggests that TGF molecules function normally as necessary mitogens for embryonic development (163, 179). Inappropriate expression in adult cells could be a step in transformation.

Nuclear Proteins

The products of five oncogenes; **myc**, **myb**, **fos**, **ski** (77), and **B-lym** (65) are known to be located in the nucleus. The expression of **c-myc**, **c-fos**, and **c-myb** appears to be dependent on the proliferative state of the cell (2, 100, 120 175). Quiescent 3T3 cells for example, have undetectable levels of **c-fos** mRNA but within 30 minutes of stimulation by PDGF (100), the levels are dramatically increased. This is only transient, and after about 2 hours the high levels disappear (120). Thus the interaction of PDGF with its receptor not only facilitates activated intracellular phosphorylation events and the breakdown of inositol lipids, but also leads to the generation of a nuclear signal to switch on **c-fos** expression. Since phosphorylation of intracellular proteins occurs within a few minutes of mitogenic stimulation , it is likely that **c-fos** expression is a direct result of these events. Like **c-fos**, the **c-myc** gene is expressed at very low levels in quiescent cells, and its transcript levels increase transiently after stimulation with PDGF, insulin, and serum (19, 22, 67, 95, 126, 175).

The roles of **c-fos** and **c-myc** gene products will be discussed in more detail below. It will be mentioned for now that since **c-myc** and **c-fos** gene expression follows a direct relationship to cell cycle, it is generally believed

that their protein products are involved in the regulation of cell division. Inappropriate expression of these nuclear proteins could keep the cell cycling even under conditions which would normally be sufficient to switch off further growth.

Molecular, Biological, and Physiological Characteristics of Proto-Oncogenes Examined in This Study

Growth Factor Related

Erb-B-1. As mentioned previously, the product of the erb-B-1 oncogene is a truncated version of the receptor for EGF (181). It is a glycoprotein with protein kinase activity and has the capability to transform cells, while the normal growth factor and receptor do not (17). The erb-B-1 oncogene protein product represents the EGF receptor short of both its large extracellular domain which binds the ligand and either 32 or 71 amino acids from its carboxy terminus (181). The transforming protein is 71 amino acids in length and includes a hydrophobic region which resides at the cell surface, a hydrophobic domain that spans the plasma membrane, and a shortened cytoplasmic domain which possesses the protein-tyrosine kinase activity (197). This

truncation could have several possible consequences, any of which may contribute to neoplastic transformation. For instance, only a small fraction of the **erb-B-1** oncogene product reaches the plasma membrane (17, 148). The remainder never leaves the golgi apparatus and retains an immature mannose-rich form. This is in contrast to the normal behavior of intact transmembrane receptors.

The EGF receptor is returned to the interior of the cell after binding ligand, a regulatory mechanism seemingly designed to protect the cell from an over abundance of stimuli. By contrast, the product of the **erb-B-1** oncogene cannot bind ligand and may be located permanantly to the surface of the cell (17, 148, 181).

The EGF receptor displays the full force of its protein kinase activity only after binding ligand. The **erb-B-1** oncogene product is presumably released from this dependence and is constitutively active (17). The kinase activities associated with the **erb-B-1** oncogene product are constitutive, and the appearance of this protein on the plasma membrane seems to be a prerequisite for transformation (14).

With respect to tumor activity, it has been reported that abnormally high levels of the **erb-B-1** oncogene were

found in 40 percent of primary brain tumors of glial origin (108). Abnormally high copy numbers of the **HER-2/neu** (c-erb-B-2) gene have been found in mammary carcinomas. This in turn has been associated with patient survival and time to relapse in diseased individuals (158).

Sis. The **sis** oncogene encodes one of the two subunits (PDGF 2-B) of platelet derived growth factor (45). Following synthesis, the 28kd product (p28**sis**) of the **sis** oncogene assembles into a homodimer and is trimmed to a smaller polypeptide (140).

The product of the **sis** oncogene may transform cells by an autocrine function. Evidence exists that some cells release a homodimer of p28**sis**, whose structure and activity resemble those of PDGF (63). Application of antibodies against PDGF to these cells arrests their growth (87). There is also reason to suspect that the **sis** oncogene product, or that of its cellular progenitor c-**sis** need not leave the cell in order to invoke neoplastic growth (13). Instead, the transforming protein may combine with a receptor while still inside the cell.

There is also the question of why the **sis** oncogene protein can transform cells, while the c-**sis** protein cannot. It is not known if there are mutations in oncogenic **sis** that

alter the capabilities of its product. Also unclear is whether or not the homodimer produced from it has abnormal activity compared to the related but different subunits of PDGF. Whether the formation of homodimer causes PDGF 2-B to be processed abnormally, or if the **sis** oncogene product acts at an anomalous site inside the cell are questions yet to be answered. It is possible that cells produce factors which cooperate with **sis** in neoplastic transformation (17). All of these issues only obviate the fact that much more needs to be done before a full understanding of the **sis** gene and its product can be obtained.

The presence of the **c-sis** gene has been demonstrated in several tumor types. Eva et al. (54) reported that cell lines from both human sarcomas and gliomas were analyzed for the presence of **sis** message. It was found to be at elevated levels in 5/6 of sarcoma cell lines and 3/5 of glioma cell lines studied. **Sis** message has also been found to be at elevated levels in the metastases of two stomach carcinomas (172).

Protein Kinases

C-Ha-ras. C-Ha-ras is a member of the ras oncogene family, and is cell cycle dependant (94). As described previously, activation of c-ras to an oncogene is accomplished by point mutations at specific sites which render its protein product oncogenic to the cell (34, 170). Mutations of this nature have been found in approximately 15 percent of sarcomas (77). Oncogenes of the ras family may be active in human carcinoma cell lines, as well as primary human tumor specimens of several sites such as colon, lung, gall bladder, urinary bladder, pancreas , rhabdomyosarcoma (42, 136,137), and in prostate cancer (186). These genes are also present in human hematopoietic neoplasias; including primary acute myelogenous leukemias, and cell lines derived from acute lymphocytic leukemias, T cell leukemias and chronic myelogenous leukemias (55, 161).

Src. The src oncogene as described above codes for a protein, which like its normal proto-oncogene counterpart, is a protein-tyrosine kinase. In order to study its mechanism of activation, c-src has been molecularly cloned from both chicken and human DNA. Nucleotide sequencing has revealed the similarities between the protein coding regions and those of v-src (171). Unlike v-src, c-src is very

complex and contains 11 introns. The exact mechanism by which RSV acquired genomic **c-src** information is unclear (196). It has been proposed that during a round of infection, a non-oncogenic RSV progenitor transduced genomic DNA after viral integration and excision, and then the introns were removed by processing. Also, it may have somehow incorporated **c-src** messenger RNA (77).

The differences between **c-src** and **v-src** have been addressed by making use of in vitro recombinants of viral and cellular genes. Results have shown that some of the amino acid changes in **v-src** are biologically important for transformation. This was also demonstrated by the fact that high levels of **c-src** expression alone did not transform cells (130). It has also been shown that if **v-src** is expressed in cells at levels comparable with those of **c-src** in normal cells, then transformation is observed (92). It may be that both qualitative and quantitative changes in **src** expression are required for transformation.

Src gene protein (p60**src**) activity is present in normal tissues where organ specific levels have been found. Jacobs et al. (91) have reported that levels of pp60**c-src** were highest in brain followed by kidney, lung, muscle, and connective tissue. It was also determined that a 4-20 fold

increase of pp60c-**src** kinase activity was present in human skin tumors compared to normal skin (7).

Nuclear Related Proto-oncogenes

Fos. The fos gene was first discovered as the oncogene of two related murine viruses that cause osteogenic sarcoma. The name **fos** refers to its origins in the FBJ and FBR osteogenic sarcoma viruses. The **fos** oncogene, like other oncogenes, causes the transformation of cells and is derived from a normal cellular gene. The cellular and viral fos genes have an interesting relationship to each other. The first 332 amino acids of **v-fos** and murine **c-fos** differ in only five positions but the remaining 49 amino acids are completely different. The 104 bases at the C-terminus of c-fos are deleted in **v-fos**, and although this changes the reading frame and alters subsequent amino acids, the mobilities of the proteins are similar (**v-fos** 55 kd, c-**fos**, 62 kd) (184).

The **fos** gene seems to serve as a kind of master switch for turning on other various genes in response to a wide range of stimuli including growth factors. **Fos** may act as a sensor which detects incoming signals at the cellular

membrane and converts them to lasting responses such as cell division and possibly memory formation (113).

The c-fos gene was recognized as a cell cycle dependent gene early after studies with it began. C-fos can be rapidly activated by the treatment of quiescent cells with PDGF, EGF, nerve cell growth factor, and serum containing growth factors (69). This led to speculation that c-fos had something to do with cellular growth control.

Studies with nerve cells revealed additional information about the activities of the c-fos gene. It was found that c-fos expression is controlled by factors which differentiate and trigger nerve cell activity. In vitro experiments have indicated that c-fos induction depends on the ability of neuroactive agents to open calcium channels (119). Calcium entry is a normal component of neuronal responses to stimulation. It was found that a dramatic increase in c-fos gene activity occurs in the brains of mice treated with metrazole, a drug which causes epilepsy like seizures (119). The synthesis of c-fos proteins was found to occur primarily in the nerve tracts stimulated by metrazole. The results suggested that the c-fos protein mediates the long term adaptation of nerve cells to metrazole stimulation.

There is now evidence for genes which seem to be directly controlled by c-fos. For example, a set of genes which code for fat cell proteins which become active when fat cells differentiate has been identified. One of these genes adipocyte P2 (aP2), was found to have a regulatory site 125 base pairs upstream from its promoter. This regulatory site binds proteins which undergo undefined changes during maturation. It is hypothesized that changes in the binding proteins mediate activation of the aP2 gene (44).

Experiments were then performed to determine whether the c-fos protein was one of these regulatory proteins. Data from immunoprecipitation analyses showed that the binding complex contains the c-fos protein itself, or at least a very related protein (44). Further studies need to be done to clarify this issue.

Site directed mutagenesis studies have been done in the c-fos promoter region. Various deletions were studied for effects on the c-fos gene's responses to various stimulatory agents. It was found that a 22 base pair region located 300 base pairs 5' to the promoter is necessary for enhanced expression of c-fos in response to serum stimulation (68, 177, 178). This region is called serum response element (SRE). These same investigators have isolated a protein

which binds specifically to the SRE. The protein appears to be necessary for c-fos response to serum stimulation, however proof that the protein directly activates c-fos remains to be obtained. SRE variants have been constructed, each with altered protein binding capabilities. It was found that the ability to stimulate transcription correllates with avidity of protein binding (68,81).

The SRE is not the only region thought to be important in c-fos gene regulation. C-fos is activated by several different stimulatory agents. It is thought that these stimulatory agents do not all act in the same manner to affect c-fos expression. The current idea is that there are multiple regulatory elements for the gene. Epidermal growth factor and phorbol esters seem to work through the SRE, but there is evidence that c-fos activation by PDGF may be mediated through a different site 25 base pairs 5' of the SRE (64, 177, 178).

Some c-fos stimulating agents use cyclic AMP as a messenger. Mutations in the SRE do not seem to affect c-fos activation by cyclic AMP (68, 177). Therefore, gene activation by cyclic AMP uses other unidentified regulatory sequences. Calcium ions which mediate c-fos during nerve cell activation may use yet another site (64, 68, 177, 178).

The different stimulatory agents which appear to use different regulatory proteins to enhance c-fos expression, induce different nuclear proteins (64, 177, 178), and these are called c-fos related antigens (FRA).

C-fos may be subject to negative regulation as well. Verma et al. (184) have shown evidence to suggest that cells may have factors which repress fos transcription, but more needs to be done before this can be fully characterized and understood.

Both the products of the v-fos (p55/v-fos) and c-fos (P62/c-fos) genes may be part of nuclear complexes (60, 184). For example, the c-fos protein complex and several FRAs bind specifically to a sequence element referred to as the HeLa cell activator protein 1 (AP-1) binding site (60). Structural studies and immunoprecipitation analyses were performed with this complex. One of the Fos-associated proteins, (p39) was found to be the protein product of c-jun (138).

The p39/jun protein is one of the major polypeptides identified in AP-1 oligonucleotide affinity chromatography extracts of cellular proteins. The preparations of AP-1 were found to contain c-fos and several FRAs (20). Some of these proteins seem to bind to the AP-1 site directly, while c-fos appears to bind indirectly through protein/protein

interactions (20). Cell surface stimulation results in an increase in c-fos and c-jun products. The products of the two genes along with several other related proteins form a complex which associates with transcriptional control elements containing AP-1 sites (20, 60, 138). This potentially can then mediate long term responses which regulate growth control and development (113).

With respect to tumor activity, there seems to be some controversy in the literature with regard to what types of neoplasms are associated with c-fos expression. It has been reported that c-fos has not been found consistently in any type of neoplasm (77). However, Slamon et al. have reported that c-fos is expressed in all tumor types including carcinomas, sarcomas, and hematopoietic malignancies (159).

Myc. The myc family of cellular proto-oncogenes contains three well defined members, c-myc, N-myc, and L-myc. The first defined and most thoroughly studied member of this family, c-myc, was identified as the cellular homolog to the transforming gene of avian transforming virus MC29 (17). The two other well characterized myc family genes N-myc and L-myc were isolated on the basis of their homology to c-myc and their frequent amplification in certain classes of human tumors. The N-myc gene was originally isolated from human

neuroblastomas, a pediatric tumor of embryonal origin that arises in the peripheral nervous system.

The **N-myc** and **c-myc** genes have a very similar overall structure, exhibit extensive homology in their coding regions, and encode similar sized nuclear proteins (41, 99). It has been confirmed that **N-myc** has transforming activity equivalent to that of **c-myc** in the rat embryo fibroblast assay (150). The **N-myc** gene has been found to be amplified in all human neuroblastomas having cytogenetic characteristics of gene amplification such as homogenously staining regions or double minutes (147). Patterns of **N-myc** amplification in neuroblastomas have been associated with tumor progression. A greater copy number of the **N-myc** gene is associated with a more advanced stage of the tumor. (152). **N-myc** activation has thusfar been found to occur only by amplification and only in a restricted set of tumors.

In addition to neuroblastomas, **N-myc** amplification has been observed in a subset of small cell lung carcinomas (SCLC) and in a few retinoblastomas (98, 106, 124). Like neuroblastomas, these tumors have neural characteristics.

Considering the oncogenic potentials and similarities of c-myc and N-myc, the reason for relatively restricted activation of the **N-myc** gene as opposed to the **c-myc** gene is

unknown. The **N-myc** gene may play a special role in certain types of neural tumors. Also, **N-myc** amplification events may be specially targeted in the precursor cells of these tumors (2).

The **L-myc** gene was isolated by two independent methods. The gene was first isolated on the basis of its amplification in a subset of SCLCs (123). The gene was independently isolated from unamplified genomes on the basis of its homology to **c-myc** and **N-myc** (2). So far, activation of the **L-myc** gene has only been observed in some SCLCs. Details with respect to the structure and transforming potential of **L-myc** call for further study, and will not be discussed here.

The expression of the **c-myc** gene has been shown to follow a fixed relation to cell cycle. Growth arrested fibroblasts (serum deprivation) in G0 show a burst of **c-myc** transcription during the G0/G1 transition when stimulated to divide by either serum addition or insulin. The **c-myc** transcript levels then decrease slowly as the cells proceed through the cell cycle, and are present at basal levels during S phase (19, 175). Nuclear run on assays with serum released G0 fibroblasts suggest that **c-myc** expression is primarily regulated post-transcriptionally, at the level of message degradation (19).

The c-myc protein product is a double stranded DNA binding protein thought to interact with other genes, perhaps those involved in cellular growth control. It is thought that the myc protein can bind to the regulatory regions of genes it controls, regulating transcription either by direct activation or by inhibition of suppression (17).

The precise function the of c-myc protein has not been elucidated. It is generally thought that its primary function is to mediate a signal(s) associated with cell division and thus, regulation of its expression is required for normal cell growth (2). Experiments with c-myc antisense RNA have shown that the ability of cells to divide can be blocked (192).

The c-myc gene has been found to be present in many types of sarcomas, carcinomas, and hematopoietic neoplasias (32, 54, 77, 172). The two most widely studied mechanisms of oncogenic activation of this gene are translocation (seen in Burkitt lymphoma), and gene amplification. In the case of Burkitt lymphoma the c-myc gene is translocated from chromosome 8 to chromosome 14 or from chromosome 8 to 22. As a result, the c-myc gene loses all or a portion of its first exon, and acquires normally unlinked sequences involved in immunoglobulin gene production (104, 121). C-myc gene

amplification has been found in many tumor types, and cell lines, including HL-60 (promyelocytic leukemia), and COLO 320 (colon carcinoma) cell lines (2, 32).

The amplified region of the **c-myc** gene has been closely studied in HL-60 cells. It has been shown that the amplified region is very large and contains multiple copies of the entire **c-myc** gene. Sequencing data indicates that amplified **c-myc** gene units or "amplicons" appear to be structurally normal (2). High levels of **c-myc** transcript have been observed in HL-60 cell lines as well as the COLO 320 line and this has been attributed to gene dosage effects. As a result of these investigations, it is commonly assumed that when high levels of **c-myc** transcript are accompanied by multiple copies, gene amplification is the cause of increased expression.

Chromatin Structure Analysis of the C-myc Gene

The chromosomes of eukaryotes replicate, undergo meiosis and mitosis, recombine, segregate, and are transcribed. The occurence of these processes is mediated through the interaction of chromosomal DNA and proteins (72). In order for these proteins to act, specific

regions of the DNA must be accesible to binding. Nuclease hypersensitive sites in chromatin are thought to be regions that are open, and will allow DNA interaction with proteins (70, 72). Therefore, it is thought that these regions are specific for regulation of genes by cis and trans acting factors. These protein accessible regions are identified by their susceptibility to cleavage with nuclease, and have been described to be twice as sensitive as other areas of chromatin (72). DNAse I hypersensitive sites are thought to represent approximately 1 percent of the entire genome (72). They were first identified by Varshavsky (182) and by Scott and Wigmore (151) who did studies with SV 40 chromatin. The presence of these sites in chromatin of mammalian cells was discovered by Wu and Elgin (195). These sites have been found in the chromatin of plants, animals fungi, and in viral genomes (72, 189). Therefore they are considered to be very important in the field of biology, and to the understanding of how genetic regulation occurs among various species of eukaryotes.

The indirect end labeling technique is most commonly used in mapping locations of DNAse I hypersensitive sites. This follows isolation of nuclei, treatment with DNAse I

and purification of the genomic DNA. Its most useful feature is that it allows mapping of DNAse I sensitive sites in a single direction (72). Regions of DNAse I sensitivity are usually the size of a nucleosomal repeat which is approximately 150-100 base pairs (72). This can make precise mapping difficult, but resolution can be improved by fine mapping techniques.

DNAse I hypersensitive sites have been associated with a wide variety of functions (72). In Saccharomyces cerevisiae, hypersensitive sites have been seen near centromeres, silencers, recombination sites, origins of replication, activation sequences, promoters, and potential sites of transcription termination (72). Therefore, these sites are probably associated with cis acting factors (72). Topoisomerases I and II, RNA polymerase II, and some transcription factors have been associated with DNAse I hypersensitive sites (72). The proteins associated with most sites in genes which have been studied have yet to be identified.

The mechanisms which are involved in the formation and maintainance of DNAse I hypersensitive sites are not clear. Because the functions of these sites are so diverse, several mechanisms are likely to be involved (72). It is thought

that interaction with trans-acting factors may be one of these mechanisms (72). The base composition of the DNA, methylation, looping, conformation, and torsional stress may also have an involvement in this process (72).

Fundamental knowledge of these principles should provide insight into molecular bases of regulation. Thus, it is a well accepted fact that DNase I hypersensitive sites represent regions where potential regulatory interactions are thought to occur. Specific DNA sequences of this nature have been shown to be located in promoter regions for such genes as globin (51), immunoglobulin (53), **c-myc** (48, 73, 155, 156), heatshock (129, 193, 194), SV 40 early region (47), and dihydrofolate reductase (154). The remainder of this review will focus on those involving the **c-myc** gene.

As previously mentioned, amplification and translocation are well known and widely studied potential **c-myc** activation mechanisms. As a result of translocation to chromosome 14 in Burkitt lymphoma, **c-myc** loses all or part of exon 1. This exon is thought to serve primarily as a regulatory region, as it is transcribed but not translated (77). Therefore, **c-myc** may be deregulated by its loss, and may be influenced by promoters of other genes proximal to its translocated site. In the case of HL-60 cells (30-50 copies of the **c-myc** gene),

it was presumed that gene dosage effects are responsible for observed increases in **c-myc** transcript. In both these instances, gross structural mutations appear to be responsible for observed changes in transcript levels. It has only been recently that we have begun to understand the effects these aberrations have on **c-myc** regulation or deregulation as the cases may be.

During the past few years, data obtained from chromatin structure analyses have demonstrated that changes in **c-myc** gene regulatory sites accompany gross structural abnormalities in both transocated and amplified states of this gene. DNAse I sensitive sites in the 5' region of exon 1 in HL-60 cells and Burkitt lymphoma cells have been investigated in 2 separate studies by Siebenlist et al. (155, 156). DNAse I hypersensitive sites in HL-60 cells were studied before and after differentiation with DMSO. Results showed that 4 DNAse I sensitive sites were present in untreated HL-60 cells (sites A, B, C, and D, figure 4). When differentiation was induced with DMSO, site B was lost. Further studies showed that the loss of this site accompanied a timely decline in **c-myc** transcript production.

In a separate study, DNase I hypersensitive sites were studied in both normal and rearranged **c-myc** alleles in a

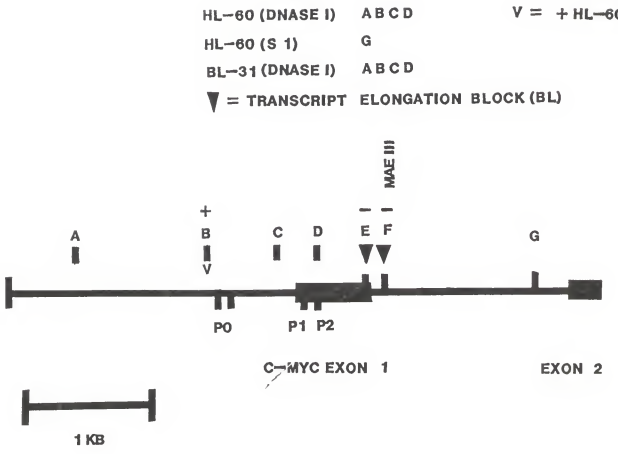


Figure 4. Summary of chromatin structure analyses previously described for the c-myc gene. Sites A, B, C, and D are DNAse I hypersensitive sites found in both HL-60 cells (Siebenlist et al (155)), and Burkitt lymphoma (BL-31) cells (Siebenlist et al. (156)). Site B (indicated by open arrow) has been described by Siebenlist et al. (155) to be involved in the maintainance of c-myc transcript production in HL-60 cells, and is therefore marked with a (+) symbol. Sites E and F (solid arows) represent transcription attenuation sites found in Burkitt lymphoma biopsies and cell lines and are marked by a (-) symbol (25, 199). Site G is an S-1 nuclease sensitive site described by Grosso and Pitot (73) in HL-60 cells.

Burkitt lymphoma cell line (BL-31) and a normal B cell line. Three different hypersensitive patterns which differed in relative band intensities were observed. These correlated with the three different transcriptional states of the **c-myc** gene examined in this study (normal B cell **myc**, unrearranged BL-31 **c-myc**, and translocated BL-31 **c-myc**). The locations of hypersensitive sites which were observed were identical to those for HL-60 cells (sites A,B,C,D) and are also shown in figure 4.

Other groups working with Burkitt lymphoma have mapped sites in the first intron and 3' region of exon 1. These were later found to be transcription attenuation sites. The loss of transcription elongation blocks at these sites is now thought to be a possible candidate for deregulation of **myc** in Burkitt lymphomas. Cesarman et al. (25) mapped a previously found DNAse I hypersensitive site (9, 10, 49) to a region near a Pvu II site in exon one. They reported that 23/26 Burkitt lymphoma cell lines and biopsies had point mutations at various sites in a specific region extending 34 bases 5' and 38 bases 3' to the Pvu II site in exon 1 (site E figure 4). These point mutations accompanied changes in transcription, namely the removal of a block to transcription mapped to the same region.

Zajac-Kaye et al. (199) noted similar findings in 5/7 Burkitt lymphoma cell lines. Their data indicated that a 20 base pair region in the first exon (site F, figure 4) was susceptible to sporadic point mutations. Mutations in this region abolished binding of a regulatory protein known to down regulate **c-myc** transcription.

Relevance to This Project

The **c-myc**, c-Ha-ras, **c-fos**, **c-sis**, **v-erb-B-1**, and **v-src** proto-oncogenes have been studied in other human tumor systems. The object of this study is to determine whether or not these genes play a significant role in the biology of chondrosarcoma and MFH. Studying transcript levels and copy numbers of these genes will offer clues to possible involvements in the pathogenesis and progression of these tumors. Furthermore, chromatin structure analysis will enhance understanding of mechanisms involved in transcript regulation.

CHAPTER 3
MATERIALS AND METHODS

Slot-Blotting of RNA and DNA

Preparation of Total Cellular RNA

Total cellular RNA was prepared from surgically obtained tumor, normal muscle, and bone marrow tissue specimens from patients treated at Shands Hospital, University of Florida. These specimens included; 20 chondrosarcomas, 23 malignant fibrous histiocytomas (MFH), 9 normal muscle, and 6 bone marrow tissue specimens. Approximately 1-3 hr after surgical removal the tissues were frozen at -70 C until use. Total cellular RNA was prepared as described by Chirgwin et al. (26). Before use, all glassware and centrifuge tubes were rendered nuclease-free with 0.1 percent diethylpyrocarbonate (DEPC) in deionized water and thouroghly dried. All stock solutions were freed of RNAse by adding several drops of 0.2 percent DEPC and subsequent autoclaving.

Tumor and normal tissues weighing 1.0-1.2 gr, or approximately 10 E6 cultured cells were placed into 10 ml of

a solution containing 4M guanidine isothiocyanate (Ultra-pure, BRL, Gaithersburg, MD), 25mM sodium citrate, pH 7.0, and 0.1 M 2-mercaptoethanol. The mixture was then homogenized using a tissuemizer (Brinkman Instruments), and 0.75 ml of 1 M acetic acid was added. The suspensions were layered into SW 50 ultra-centrifuge tubes (Beckman instruments) containing a 1.5 ml pad of 5.7 M cesium chloride. The samples were centrifuged for 16 hr at 20 C and 35,000 rpm.

Following centrifugation, the pellets were resuspended in 1.0 ml DEPC treated, sterile H20, and extracted once with 25:25:1 phenol:chloroform: isoamyl alcohol. The RNA was then precipitated by adding 100 ul of 4 M potassium acetate, and 2.5 ul ethanol. Recovery of the RNA was accomplished by centrifugation at 10,000 rpm for 20 min at 4 C. The pellets were resuspended in 0.2 mM EDTA, visualized on formaldehyde agarose gels (as described for northern blotting below), quantitated by absorbance at 280 nm, and stored in aliquots at -70 C.

Preparation of Genomic DNA

Genomic DNA was prepared from the same tumor, muscle, and bone marrow tissue specimens as described above. Tissues were frozen in liquid nitrogen and ground to a fine powder with a morter and pestal. This powder, or approximately 10 E6 cells was suspended in 9.2 ml of STE (100mM NaCl, 20mM Tris, pH 8.0, 10mM EDTA). Two hundred ul of 0.5M EDTA and 200ul of proteinase K (10mg/ml) were then added, and the mixture was incubated overnight at 65 C.

Following incubation, the mixture was extracted once with an equal volume of phenol, once with an equal volume of phenol/chloroform-isoamyl alcohol (24:1), and finally once again with an equal volume of chloroform-isoamyl alcohol. The DNA was recovered by spooling after the addition of an equal volume of isopropanol, and resuspended in 10mM Tris, 1mM EDTA.

RNA Slot-Blotting

Quantites of 10, 5, and 2.5 ug of total cellular RNA were denatured in a solution containing 100 ul of water and 300ul of an RNA denaturant solution containing 6.15 M formaldehyde and 10X SSC (sodium chloride, sodium citrate). The samples were incubated at 65 C for 15 min and loaded onto a mini-

fold II slot-blotter (Schleicher & Schuell, Keene, NH) using procedures described by Wahl (187) . The slot-blotter contained a Nitro-Plus 2000 filter (Micron Separation Sciences) onto which the RNA was blotted.

DNA Slot-Blotting

Quantities of 20, 10, and 5 ug of DNA were suspended in 400 ul of 10mM Tris, 1mM EDTA, pH 7.0, and 40 ul 3M NaOH, then incubated at 65 C for 45 min. After incubation, the samples were cooled on ice, 400 ul of 2 M ammonium acetate were added, and the samples were loaded onto a minifold II slot-blotter as described above.

After slot-blotting, the filters were air dried to completion, then baked in a vacuum oven at 80 C for 2 hr. The blots were then incubated at 42 C overnight in a pre-hybridization solution (5 ml/ 100 square cm) containing 5X SSC, 10X Denhardt's solution (0.2 percent ficoll, 0.2 percent polyvinylpyrrolidone (PVP), 0.2 percent bovine serum albumin (BSA)), 0.05M sodium phosphate pH 6.7, 500ug/ul sonicated, denatured salmon DNA, 5 percent dextran sulfate (Pharmacia Chemical Co., Piscataway,NJ), and 50 percent formamide (112, 160).

Preparation of Radiolabeled Probes

Descriptions, sources, and methods of labeling for all probes used in slot-blot, Southern blot, northern blot, and chromatin structure analysis are summarized in Table 1. Figure 5 shows locations of the different **c-myc** probes as well as other probes located on chromosome 8 used in mapping and dilutional analysis of **c-myc** amplicons in MFHs. Restriction maps of all other probes in Table 1 are shown in figure 6.

Nick Translated Probes

Probes were nick translated by adding 250 ng of DNA to a reaction mixture which contained 80 uCi 32P (dATP), 5.0 ul of 10X (dCTP, dGTP, dTTP), 1.25 ul of 1mg/ml bovine serum albumin (BSA), 5 ul of nick translation buffer (0.5 M Tris HCL, pH 7.8, 0.1 M 2-mercaptoethanol, and 0.05 M MgCl2), 1.5 ul DNAse I/Polymerase I (BRL, Gaithersburg, MD), and deionized H20 to a final volume of 25 ul. The reaction was run at 15 C for 45 min. Labeled DNA was separated from unincorporated nucleotides using a Biogel A-15m (Biorad, Rockville Center, NY) column.

Table 1. Summary of probes used in slot-blotting, Southern blotting, northern blotting, and chromatin structure analyses.

Probe Name	Source*	Use**	Reference	Method of Labeling+	Description
C-myc	1	1,4	1	N.T	9.0 kb Eco R 1/ Hind III human genomic fragment cloned into PBR 322
C-Ha-ras	1	1	137	N.T.	6.4 kb Bam H1 human genomic fragment cloned into PBR 322
C-fos	1	1	36	N.T.	6.4 kb Xho I/ Nco I human genomic fragment cloned into PBR 322
C-sis	2	1	71	N.T.	1.0 kb human genomic fragment cloned into pSP 6
V-erb-B-1	1	1	108	N.T	1.7 kb Pvu II/ Sst I genomic fragment from avian erythro-blastosis virus
V-src	1	1	37	N.T.	800 bp Pvu II genomic fragment from avian sarcoma virus prague A strain

Table 1. Continued.

Probe Name	Source*	Use**	Reference	Method of Labeling+	Description
Beta-actin	1	1	28	N.T.	800 bp Nco I/Taq I genomic fragment from chicken beta-actin gene
TK (pTK 11)	3	1,2	21	N.T/P.E.	1.25 kb Sma I Bam H I human genomic fragment
pGEM-H MYC	4	2,3,4	188	N.T	1020 bp Pst I human cDNA fragment cloned into pGEM 1
p380-8A	5	4	78	N.T.	1.8 kb Sal I/SstI human genomic fragment cloned into puc 19
Carbonic Anhydrase (H 25-3.8)	6	4	183	N.T.	3.8 kb Eco R I human genomic fragment cloned into PBR 325
Thyro-globulin (HT .96)	6	4	3	N.T.	960 bp Pst I human cDNA cloned into puc 8

Table 1. Continued.

Probe Name	Source*	Use**	Reference	Method of Labeling+	Description
Beta-actin	7	2	75	P.E.	2.0 kb Bam H1 human cDNA fragment
PMC 41	8	5	73	N.T.	1.6 kb Cla I/ Eco RI human genomic fragment cloned into PBR 322
C-myc Sca/Xho I	9	6	1	P.E.	355 bp Sca I/ Xho I human genomic fragment

* Sources: 1) Oncor, Inc, Gaitersburg, MD, 2) Oncogene Sciences, Mineola, NY, 3) Dr. Harvey Bradshaw, 4) Dr. Ken Soprano, 5) Dr. Carlo Croce, 6) American Type Culture Collection, Rockville, MD, 7) Dr. Larry Kedes, 8) Dr. Robert Gallo, and 9) Made from **c-myc** plasmid obtained from Oncor, Inc. (above)

** Uses: 1) Slot-blot hybridization, 2) northern blot hybridization, 3) titration of **c-myc** gene copy number in MFHs, 4) Mapping of **c-myc** amplicons in MFHs, 5) mapping DNAse I hypersensitive sites in cell lines from the 3' direction, and 6) mapping DNAse I hypersensitive sites in cell lines from the 5' direction (fine mapping analysis).

\+ Method of labeling: N.T. = nick translation, P.E. = random primer extension.

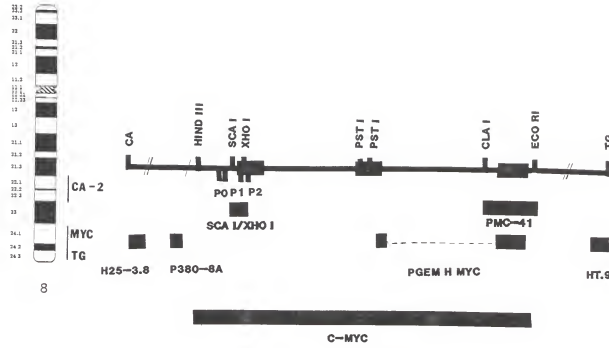


Figure 5. Locations of the different **c-myc** probes as described in table 1. Also shown are other probes located on chromosome 8 which were used in mapping of **c-myc** amplicons in MFHs.

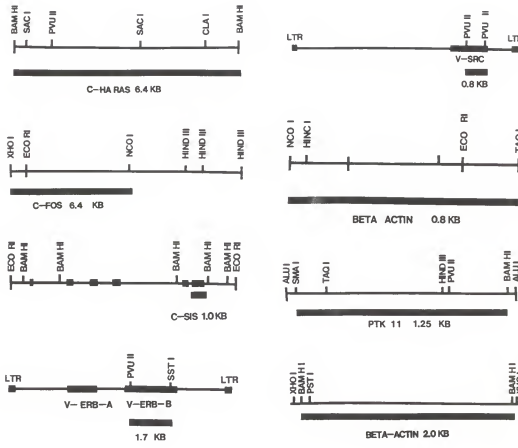


Figure 6. Restriction maps of non-c-myc/chromosome 8 probes as described in table 1.

Probes Labeled by Random Primer Extension

Two hundred ng of DNA were denatured at 90 C for 2 min. After denaturation, five ul of 5X primer extension buffer (1M hepes, pH 6.6, 25mM MgCl2, 50 mM 2-mercaptoethanol, 0.25 M Tris HCL, pH 8.0, 0.1 mM dCTP/dGTP/dTTP, 2mg/ml BSA, 15 mg/ml primer), 5 units Klenow (BRL, Gaithersburg, MD), 100 uCi 32P (dATP) and H20 were added to a final volume of 25 ul. This reaction mixture was allowed to sit at room temperature for 16 hr. Unincorporated nucleotides were separated from labeled DNA as described for nick translations.

Hybridization of Slot-Blots

Slot- blots were hybridized at 42 C with 3.0 X 10 E6 cpm (1.0 X 10 E8 cpm/ug) of probe for at least 20 hr in 15 ml of hybridization solution containing 5X SSC, 1X Denhardt's solution, 0.02 M sodium phosphate, pH 6.7, 100ug/ml sonicated, denatured salmon DNA, 10 percent dextran sulfate, 50 percent formamide, and 6 percent water (112).

Post hybridization washes were carried out by washing the filters twice for 15 min at room temperature with 2X sodium chloride, sodium phosphate, EDTA (SSPE), 0.1 percent

sodium dodecyl sulfate (SDS). The blots were then washed twice again at 50 C with 0.1X SSC, 0.1 percent SDS for 30 min each and exposed to preflashed X-ray film for 36-48 hr at 70 C with intensifying screens.

Slot-blots were rehybridized after treatment of the membrane to remove bound probe. This was accomplished by pouring 1 liter of 0.1 X SSPE, 0.1 percent SDS heated to 90 C over the blots. The solution was then cooled to 70 C and removed. (187)

Southern Blot Analysis

Genomic DNA was prepared as described above from tissue samples and cell lines. Aliquots of DNA were restricted with appropriate restriction endonucleases and electrophoresed through 0.8 percent agarose gels (65 volts, 16 hr). Digested DNA was then transferred to Zetabind (AMF Cuno, Meriden, CT), pre-washed in 0.1 X SSC, 0.1 percent SDS at 65 C for 1 hr, and hybridized (2 X 10 E6 cpm/ml/10 E8 cpm/ug) using pre-hybridization and hybridization conditions previously described for slot-blotting (112, 160). Post hybridization washes were performed by washing the membrane at room temperature for 15 min, once with 2 X SSC,

0.1 percent SDS, and once again with 0.1 X SSC, 0.1 percent SDS. The blots were then washed twice for 30 min at 60 C, with 0.1 X SSC, 0.1 percent SDS and exposed to X-ray film at -70 C with intensifying screens. Rehybridization of the blots was accomplished after removal of bound probe. This removal process consisted of washing the blots in 0.1 X SSC, 0.5 percent SDS at 80 C for 15-20 min.

Northern Blot Analysis

Total cellular RNA was prepared from cell lines as described for slot-blotting. RNA was denatured at 55 C for 15 min in an RNA denaturant containing 5ul 10X MOPS, 8.75 ul 37 percent formaldehyde, 25 ul formamide (ultra-pure BRL, Gaithersburg, MD), and water to a final volume of 50 ul. After denaturation, 10 ul of RNA formaldehyde loading dye was added (500 ul formamide, 162 ul 37 % formaldehyde, 350 ul glycerol, 100 ul 10X MOPS, bromophenol blue). The samples were electrophoresed through 1.2 percent formaldehyde agarose gels (139). The gels were prepared by melting 4.2 gr agarose in 304.5 ml water. After cooling, 35 ml MOPS and 10.5 ml formaldehyde were added. Samples were

electrophoresed in running buffer which consisted of 1X MOPS and 10 percent formaldehyde (volume/37%) at 120 volts for 3-3.5 hr.

After electrophoresis, the gels were rinsed several times in deionized water, then soaked in 10X SSC for 45 min. Blotting stacks were assembled as for Southern blotting. Overnight transfers to Zetabind membranes were completed in 20X SSC. Blots were pre-washed in 0.1 X SSC, 0.5 percent SDS at 65 C for 1 hr. Pre-hybridization and hybridization conditions (2.0 X 10 E6 cpm/ml/ 10 E8 cpm/ug), as well as post-hybridization washes and rehybridization procedures were identical to those described for Southern analysis.

Chromatin Structure Analysis

Cell Lines Used in Chromatin Structure Analysis

UR-HCL-1. The UR-HCL-1 cell line is a human MFH tumor cell line obtained from ATCC.

P3C. The P3C cell line is an MFH tumor cell line obtained from Dr. Byron Croker, Department of Pathology, University of Florida. The cell line was made by culturing

an MFH from a patient treated at Shands Hospital, University of Florida.

ST 486. The ST 486 cell line is a Burkitt lymphoma cell line obtained from ATCC. This cell line was used as a positive control for chromatin structure analyses, as DNAse I hypersensitive patterns for Burkitt lymphoma c-myc have been described (156).

HFF. The HFF normal human fibroblast cell line was obtained from Dr. Kenneth Rand, Department of Pathology, University of Florida.

Preparation of Nuclei

Cells were grown in Dulbeccos MEM (minimal essential medium) (Gibco, Gaithersburg, MD), supplemented with 10 percent fetal bovine serum (Gibco, Gaithersburg, MD). Nuclei were isolated from dividing cells (approximately 2.0 X 10 E8). The cells were washed in 100 ml 1X phosphate buffered saline (PBS), and centrifuged at 2,000 rpm, for 3 min at 4 C. The pellet was resuspended in 10 ml 1X RSB (10mM Tris, pH 7.4, 10mM NaCl, 3mM Mg Cl2), 0.5 percent

nonidet P40 (NP 40), 10 ul 0.1M phenyl methyl sulfate (PMSF), then incubated on ice (0 C) for 5 min. The nuclei were recovered by centrifugation at 2,000 rpm at 4 C for 3 min. The pellet was then washed 3 times with 100, 50 and 20 ml of 1X RSB followed by centrifugation at 2,000 rpm at 4C for 3 min.

The nuclei were then resuspended in 1X RSB and digested with varying concentrations of DNAse I (Boerhinger Mannheim) for 10 min at 37 C. Controls were 0 ug/ml DNAse I incubated at both 0 and 37 C.

Isolation of Genomic DNA From DNAseI Treated Nuclei

After digestion with DNAse I, the samples were placed on ice and 1/10 volume 0.25M EDTA was added along with 1/20 volume 10 percent SDS and 1/20 volume proteinase K. After incubation overnight at 37 C, the samples were extracted with an equal volume phenol, an equal volume phenol/24:1 chloroform-isoamyl alcohol, then a third time with an equal volume of 24:1 chloroform - isoamyl alcohol. DNA was precipitated by adding 4M potassium acetate to a final concentration of 0.3M, and the addition of 2-3 volumes cold 95 percent ethanol. After precipitation at 20 C overnight,

DNA was recovered by centrifugation at 10,000 rpm at 4 C for 15 min. The pellet was resuspended in 300 ul 50mM Tris, pH 8.0, 10 mM EDTA. RNAse A (1mg/ml) was added to a final concentration of 50 ug/ml and incubated overnight at 37 C. The mixture was then phenol/chloroform extracted, and the DNA precipitated as described above. The final DNA pellet was resuspended in 10mM Tris, 1mM EDTA.

Aliquots of DNA from each of the cell lines were restricted with either Eco R1 (mapping of DNAse I hypersensitive sites from a 3' direction (pmc 41 probe)), or Sca I (mapping of these sites from a 5' direction (Sca I/Xho I fragment probe)), and analyzed using Southern blotting and hybridization methods described above. The mapping of DNAse I hypersensitive site locations was accomplished through the use of the indirect end labeling technique. This technique allows mapping of the DNAse I hypersensitive sites in one direction, and is described in figure 7 using restriction with Eco R1 and hybridization with pmc 41 as an example.

Chromatin Structure/ Fibroblast Cell Synchrony Experiment

HFF cells were grown to 70-80 percent confluency in Dulbeccos MEM supplemented with 10 percent fetal bovine

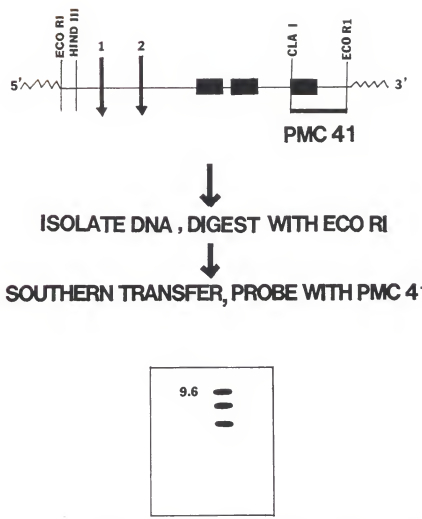


Figure 7. Illustration of the indirect end labeling technique. This technique allows 5' or 3' orientation of the locations of DNAse I hypersensitive sites. It always yields pieces of DNA which have a restriction site on one side, therefore allowing analysis of DNA segments in one direction. Various concentrations of DNAse I are used to allow partial digestion. If DNAse I cut at sites 1 and 2, analysis by this method would yield three bands on a Southern blot. A main band of 9.6 kb which corresponds to the Eco R1/Eco R1 fragment would be present along with bands of sizes corresponding to the lengths of the DNAseI cleavage sites to the 3' ECO R I site.

serum. At this confluency level, cells were actively cycling (confirmed by Northern blot hybridization with the TK probe data shown below). The cells were then made quiescent by the addition of MEM containing 0.1 percent serum, and subsequent incubation at 37 C for 3 days.

MEM supplemented with 10 percent fetal bovine serum was then added to release the cells. Nuclei isolation/DNAse I treatment (as described above) and RNA isolation procedures (as described above) were conducted at 0 hr (G0), 0.5 hr, 1 hr, 2 hr, 3 hr after serum release, and during log phase growth. DNAse I hypersensitive sites were evaluated by Southern blot hybridization procedures using the pmc 41 probe as described above. RNA samples were analyzed for c-myc, TK, and actin transcript levels using Northern blot procedures as previously described.

Strategy for Fine Mapping DNAse I Hypersensitive Sites 3' to C-Myc Exon 1

An overall scheme for fine mapping of DNAse I hypersensitive sites from a 5' direction is shown in figure 8. Selected restriction enzyme sites in the region of a DNAse I hypersensitive site 3' to c-myc exon 1 (discussed

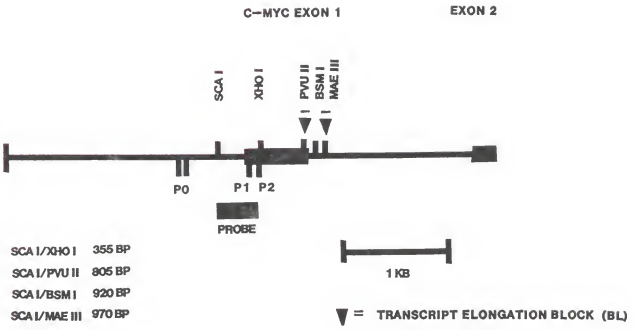


Figure 8. Location of the Sca I/Xho I probe and other restriction sites in and around c-myc exon 1 used in fine mapping of DNAse I hypersensitive sites from the 5' direction in P3C and HFF cell lines. These sites were used to generate P3C DNA fragments of known sizes, which were internal size markers on the mapping blot (see materials and methods). Also shown are the c-myc promoters (P0, P1, and P2), and known c-myc transcript elongation block sites which are indicated by solid arrows.

below) were chosen to generate fragments used as internal gel markers to map DNAse I hypersensitive sites in this region.

First, P3C genomic DNA was restricted to completion with Sca I (Boerhinger Mannheim). Five ug aliquots of this DNA were then restricted a second time with either Pvu II (BRL, Gaithersburg, MD), Bsm I (New England Biolabs), or Mae III (Boehinger Mannheim).

Bsm I cut a single time within the **c-myc** gene, therefore the restriction reaction was allowed to go to completion overnight at 65 C. Pvu II and Mae III cut at multiple sites within the **c-myc** gene. Reactions were therefore controlled to prevent complete digestion of the DNA. Partial digestion of DNA with an enzyme which cuts at multiple sites between a desired site and Sca I will yield band sizes corresponding to distances between the desired site and the Sca I site.

Five ug aliquots of Sca I restricted P3C DNA were restricted with various concentrations of Pvu II (37 C) and Mae III (55 C) for 30 min reaction times. Concentrations which yielded optimum visualization of the desired marker band sizes were used to restrict P3C DNAs for use as markers in mapping analyses.

DNAse I treated DNA samples (samples with optimum visualization of DNAse I generated bands from previous analysis) from P3C (0.2 and 0.5 ug/ml) and HFF (0.5 ug/ml) cell lines were restricted with Sca I. Five ug aliquots of these samples along with marker DNAs generated as described above were electrophoresed in 1.5 percent agarose gels (65 volts, 20 hr), and blotted onto Zetabind. Lambda DNA digested with Eco R 1 and Hind III was run on either side of the gel to assure that the gel ran evenly. Prehybridization, hybridization (2.0 X 10 E 8 cpm/ml/ 10 E8 cpm/ug), and washing conditions were identical to those described for Southern blotting.

Polyacrylamide Gel Electrophoresis (PAGE)

The discontinuous system for PAGE as described by Laemmli (101) was used in this analysis. Stacking gels were 4 percent acrylamide (total) in 0.125 M Tris-HCl, pH 6.8, and 0.1 percent SDS. Separating gels were 8.5 percent total acrylamide in 0.375 M Tris-HCL, pH 8.8, and 0.1 percent SDS. Both gels were cross-linked with 2.7 percent bis acrylamide, and polymerization was catalyzed with 0.005 percent TEMED, and 0.05 percent ammonium persulfate.

Twenty ug of protein were combined with an equal volume of treatment buffer (0.125 M Tris-HCL, pH6.8, 4 percent SDS, 20 percent glycerol, and 10 percent 2-mercaptoethanol), incubated at 90 C for 1.5 min, ice-quenched, then loaded onto the gels. Molecular weight markers ranging from 31,000 to 200,000 daltons (Biorad) were loaded as well.

A tank buffer which consisted of 25 mM Tris-HCL, pH 8.3, 0.192 M glycine, and 0.1 percent SDS was used as a running buffer. Gels were electrophoresed in a Hoefer SE 600 vertical slab unit at 30 ma/1.5 mm gel thickness.

Western Blotting and Immunoperoxidase Assay

Western blotting of proteins was carried out at 0.6 amps for 45 min at 4 C. Proteins were blotted onto 0.2 um pore size nitrocellulose (Schleicher & Schuell, Keene, NH) using methods described by Towbin (176). Transfer was carried out using a Hoeffer TE 52 Transphor unit. Following transfer, the blots were air-dried, then incubated for 3 hr in PBST (1X phosphate buffered saline (PBS), 0.05 percent Tween 20) and 2 percent BSA. An anti- human **c-myc** monoclonal antibody (HL-40) (IGG 1, ascites purified by protein A column) obtained from Dr. Henry Neiman was then diluted (0.1 mg/ml) in PBST, 2

percent BSA, added to one blot, and allowed to incubate at room temperature for 1 hr with light agitation. As a control for non-specific binding, an identical blot was incubated with an anti-met 72 monoclonal antibody (K 88. 151. G 127) (IGG 1, ascites purified by protein A column), (0.01 mg/ml in PBST, 2 percent BSA) obtained from Dr. Arthur Kimura. The blots were then washed 3 times for 5 min each with PBST. This was followed by incubation with a 1 ug/ml solution of a horseradish peroxidase conjugated goat-anti-mouse Ig (Southern Biotechnology Associates) diluted in PBST, 2 percent BSA for 1 hr at room temperature. Blots were then washed 3 times with PBST as before, and incubated with a 180 ug/ml solution of the substrate, diaminobenzoate (DAB), in PBST, and 0.01 percent H202 for 2-3 minutes. The reaction was stopped with excess H20. Quantification of c-myc protein bands was carried out by reflectance densitometry.

As a control for quantification, a third identical blot was stained with a 0.1 percent solution of india ink in PBST for 1 hr at room temperature, then destained with PBST until the desired resolution was achieved.

CHAPTER 4
RESULTS

Quantitation of Moderately Degraded RNA Using the Slot-Blotting Technique

The relationship between RNA degradation and accuracy of quantitation was evaluated because in many instances tumor tissues were not immediately (1-3 hr) available for processing after surgical removal, and message degradation occurs rapidly. Intact total cellular RNA from HL-60 cells was degraded in 0.2 N NaOH at 0, 0.5, 1,2,5, and 30 minute intervals. After evaluation by formaldehyde gel electrophoresis using procedures previously described (northern blotting section), (figure 9), the sample at 0 minutes showed completely intact RNA, the samples at 0.5, 1, 2, and 5 minutes, moderately degraded RNA, and at 30 minutes the RNA was extensively degraded. Analysis of these samples by slot-blotting (figure 10) demonstrated that moderately degraded RNA as shown in lanes B, C, D and E, is as sensitive to quantitative changes as intact total cellular RNA (lane A). Extensively degraded RNA shown in

Figure 9. Formaldehyde gel electrophoresis of 10 ug of HL-60 RNA following alkaline degredation with 0.2 N NaOH and neutralization with 0.1 M Tris, Ph 7.5 after (a) 0 min (b) 0.5 min (c) 1 min (d) 2 min (e) 5 min and (f) 30 min. Lane a shows totally intact RNA, lanes b, c, d, and e intermediate degredation, and lane f, extensively degraded RNA.

Densitometer readings for slot-blot of alkaline degraded total cellular RNA from HL-60 cells as shown above.

A	8.71 E5
B	8.64 E5
C	9.02 E5
D	8.41 E5
E	8.66 E5
F	1.09 E5

Figure 10. Slot-blot of alkaline degraded HL-60 total cellular RNA hybridized with the **c-myc** probe (3.0 X 10 E6 cpm / 1 X 10 E 8 cpm/ug). Densitometric values (peak areas sq um/ug) correspond to hybridization signals for 2.5 ug of RNA neutralized after (a) 0 min (b) 0.5 min (c) 1 min (d) 2 min (e) 5 min and (f) 30 min. Densitometric values (shown below blot) for lanes A-E indicated that moderately degraded RNA was as reliably quantitated by slot-blotting as totally intact RNA. Extensively degraded RNA (lane F) was not, and no samples showing this level of degredation were used in these analyses.

lane F was unacceptable for analysis because it could not be evaluated for gene expression as quantitatively as total cellular RNA. Also, the hybridization signal intensity of the extensively degraded RNA was just below the linear range for this system which was determined to be 1.10 X 10 E5-2.2 X 10E 6 square um/ug RNA (166). No patient samples showing this level of degradation were used. All RNA samples used in this study conformed to the criteria for moderate degredation after visualization by formaldehyde gel electrophoresis (figure 11).

RNA from 20 chondrosarcomas, 23 malignant fibrous histiocytomas, 9 normal muscle (normal non-proliferating mesenchymal tissue), and 6 bone marrow (normally proliferating mesenchymal tissue) tissues were screened for proto-oncogene transcript levels and gene copy numbers of c-myc, c-Ha-ras, c-fos, c-sis, v-erb-B-1, and v-src. These genes were studied because of previous associations with sarcomas in humans and other animals. The v-erb-B-1 and v-src gene probes were used because c-erb-B-1 and c-src were not available.

Hybridization signal intensities from these blots were normalized to those of beta-actin and thymidine kinase (TK). Beta-actin is a constitutively expressed non-cell-cycle

Figure 11. RNA formaldehyde gel electrophoresis of total cellular RNA from 6 MFHs. These samples illustrate typical moderate degredation seen with RNA samples extracted from tumor and normal tissues.

dependent gene (75, 134), and was used to normalize hybridization signal intensities for c-sis, v-erb-B-1, and v-src, which also are non-cell-cycle-dependent. Hybridization signal intensities of cell-cycle dependent genes c-myc, c-Ha-ras, and c-fos were normalized to TK, also a cell-cycle-dependent gene (93). Ratios of TK/actin were taken as a molecular measurement of cell division.

The extent of hybridization for slot-blots was estimated by densitometric scanning of the x-ray film (Bio-rad video densitometer). Results were reported as the logarithm of the peak areas for 10, 5, and 2.5 ug for RNA, and 20, 10, and 5 ug for DNA. The mean values for the 3 aliquots of each RNA and DNA sample were calculated and used to determine a gene:actin and gene:TK ratio (tables 2-13). All DNA values are normalized to actin as are RNA values for c-sis, v-erb-1, and v-src. C-myc, c-Ha-ras, and c-fos RNA levels are normalized to TK. Significant differences between any two groups were determined with Students T test.

RNA Slot-Blot Results

Thymidine kinase, c-myc, c-Ha-ras, and c-fos are undetectable in muscle using this method of detection

Table 2. C-myc, c-Ha-ras, and c-fos:TK ratios from slot-blot analyses of total cellular RNA from normal muscle and bone marrow tissues. Also shown are TK:actin ratios which were used as molecular measures of cell cycle.

SAMPLE	c-myc TK	c-Ha-ras TK	c-fos TK	TK actin
MUSCLE				
MFH patients				
MM-1	*	*	*	*
MM-2	*	*	*	*
MM-3	*	*	*	*
MM-4	*	*	*	*
MM-5	*	*	*	*
CHONDROSARCOMA patients				
MC-1	*	*	*	*
MC-2	*	*	*	*
MC-3	*	*	*	*
MC-4	*	*	*	*
BONE MARROWS				
BM-1	1.18	1.28	1.05	0.909
BM-2	0.945	1.15	0.935	1.01
BM-3	0.848	0.831	0.834	1.02
BM-4	0.804	0.972	0.935	1.13
BM-5	0.551	1.05	1.08	1.28
BM-6	0.957	0.813	0.968	1.15

* C-myc, c-Ha-ras, c-fos, and TK were undetectable in normal muscle tissues by these analyses.

Table 3. C-sis, v-erb-B-1, and v-src:actin ratios from slot-blot analyses of total cellular RNA from normal muscle and bone marrow tissues.

SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
MFH patients			
MM-1	0.970	0.770	0.926
MM-2	0.989	0.882	1.02
MM-3	0.963	0.842	0.973
MM-4	0.904	0.835	1.00
MM-5	0.911	1.02	1.07
CHONDROSARCOMA patients			
MC-1	0.933	0.897	0.949
MC-2	0.947	0.765	0.763
MC-3	0.897	0.981	1.06
MC-4	1.14	0.949	0.893
Bone marrows			
BM-1	1.15	1.28	1.18
BM-2	1.10	1.18	0.989
BM-3	1.09	0.863	0.876
BM-4	0.925	1.10	1.26
BM-5	1.16	1.22	0.998
BM-6	1.02	1.27	1.07

Table 4. c-myc, c-Ha-ras, and c-fos:actin ratios from slot-blot analyses of genomic DNA from normal muscle and bone marrow tissues. Also shown are TK:actin ratios.

SAMPLE	c-myc actin	c-Ha-ras actin	c-fos actin	TK actin
MFH patients				
MM-1	1.06	1.01	0.964	0.810
MM-2	1.03	1.01	0.898	0.951
MM-3	1.27	1.03	0.876	0.920
MM-4	0.948	0.958	0.979	0.944
MM-5	0.964	0.964	0.918	0.996
CHONDROSARCOMA patients				
MC-1	1.21	1.17	0.952	0.955
MC-2	1.02	1.06	0.842	0.747
MC-3	1.22	1.05	0.868	1.10
MC-4	1.03	1.21	0.952	0.924
Bone marrows				
BM-1	0.746	0.918	0.858	0.903
BM-2	1.16	0.990	1.21	1.21
BM-3	0.853	1.08	0.975	1.02
BM-4	0.743	0.792	0.743	0.847
BM-5	1.09	0.903	1.13	1.27
BM-6	0.786	0.731	0.786	0.897

Table 5. C-sis, v-erb-B-1, and v-src:actin ratios from slot-blot analyses of genomic DNA from normal muscle and bone marrow tissues.

SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
MFH patients			
MM-1	0.979	0.907	0.927
MM-2	0.962	1.00	1.04
MM-3	1.01	1.03	1.01
MM-4	0.876	0.856	0.825
MM-5	0.933	0.989	0.893
CHONDROSARCOMA patients			
MC-1	0.856	1.06	0.968
MC-2	0.852	0.974	0.888
MC-3	0.953	1.05	0.108
MC-4	0.925	0.973	0.957
Bone marrows			
BM-1	1.06	1.00	0.940
BM-2	1.05	1.14	1.13
BM-3	0.829	0.846	0.919
BM-4	0.861	0.757	0.822
BM-5	1.18	1.19	0.894
BM-6	0.882	1.09	0.844

Table 6. C-myc, c-Ha-ras, and c-fos:TK ratios from slot-blot analyses of total cellular RNA from chondrosarcomas. Also shown are TK:actin ratios which were used as molecular measures of cell cycle.

CHONDROSARCOMA SAMPLE	c-myc / TK	c-Ha-ras / TK	c-fos / TK	TK / actin
CS-1	0.693	0.522	1.02	1.16
CS-2	1.02	0.971	1.06	0.752
CS-3	0.911	1.64	0.934	0.596
CS-4	0.531	0.218	0.292	1.16
CS-5	0.452	0.594	2.49	1.19
CS-6	0.468	0.195	1.60	2.19
CS-7	1.41	1.02	1.35	0.608
CS-8	1.03	0.575	1.16	0.861
CS-9	1.19	0.887	1.43	0.653
CS-10	0.804	0.666	1.06	1.50
CS-11	2.49	1.62	0.982	0.370
CS-12	0.726	0.383	1.14	0.698
CS-13	2.17	0.502	0.894	0.839
CS-14	1.80	1.90	0.976	0.301
CS-15	0.812	0.353	1.20	1.52
CS-16	**	0.466	0.927	2.47
CS-17	0.416	0.468	1.00	2.56
CS-18	1.10	1.46	0.760	0.996
CS-19	2.63	0.233	0.934	0.331
CS-20	1.23	1.06	1.54	0.536

** C-myc was not detectable.

Table 7. C-sis, v-erb-B-1, and v-src:actin ratios from slot-blot analyses of total cellular RNA from chondrosarcomas.

CHONDROSARCOMA SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
CT-1	2.42	0.821	0.993
CT-2	1.87	1.37	1.20
CT-3	0.637	0.060	0.841
CT-4	2.60	2.70	0.798
CT-5	1.22	2.57	0.888
CT-6	2.29	0.928	0.783
CT-7	0.180	2.31	1.01
CT-8	0.604	0.714	0.863
CT-9	6.08	1.86	0.697
CT-10	2.32	1.26	1.29
CT-11	0.130	1.28	0.900
CT-12	2.14	1.15	0.700
CT-13	0.457	0.979	0.801
CT-14	0.135	0.561	0.777
CT-15	3.38	0.822	1.33
CT-16	0.247	0.549	1.11
CT-17	1.79	0.511	0.890
CT-18	1.97	0.448	0.697
CT-19	0.637	0.688	0.770
CT-20	0.148	0.605	0.838

Table 8. C-myc, c-Ha-ras, and c-fos:actin ratios from slot-blot analyses of genomic DNA from chondrosarcomas. Also shown are TK:actin ratios.

CHONDROSARCOMA SAMPLE	c-myc actin	c-Ha-ras actin	c-fos actin	TK actin
CS-1	0.980	0.825	0.948	0.951
CS-2	0.757	0.933	1.20	1.06
CS-3	0.878	0.929	0707	0.982
CS-4	0.955	0.700	0.642	1.06
CS-5	0.922	1.16	1.14	1.04
CS-6	0.775	0.769	0.656	0.973
CS-7	1.03	0.735	0.816	0.974
CS-8	0.933	0.698	0.872	0.903
CS-9	1.14	0.901	1.18	1.24
CS-10	1.23	1.35	0.956	1.23
CS-11	1.24	0.756	0.783	1.08
CS-12	0.993	0.868	1.03	0.833
CS-13	1.12	1.20	1.33	1.19
CS-14	1.08	0.800	1.24	1.04
CS-15	1.03	0.793	1.25	0.833
CS-16	0.885	1.23	0.799	1.19
CS-17	1.25	1.05	1.32	1.04
CS-18	1.09	1.04	0.774	0.879
CS-19	1.16	0.901	1.31	1.18
CS-20	1.05	1.14	1.09	1.17

Table 9. C-sis, v-erb-B-1, and v-src:actin ratios from slot-blot analyses of genomic DNA from chondrosarcomas.

CHONDROSARCOMA SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
CS-1	1.25	0.933	0.749
CS-2	0.925	0.995	0.766
CS-3	1.28	0.846	1.28
CS-4	1.14	0.997	0.803
CS-5	1.36	1.38	1.35
CS-6	1.31	1.34	1.37
CS-7	1.24	1.24	1.22
CS-8	1.12	0.976	1.00
CS-9	1.17	1.06	1.24
CS-10	1.04	0.842	1.39
CS-11	1.22	0.784	1.25
CS-12	1.18	1.26	1.30
CS-13	1.29	1.29	1.32
CS-14	1.14	0.944	1.16
CS-15	1.23	0.898	1.19
CS-16	1.31	0.910	1.31
CS-17	1.39	0.805	1.20
CS-18	1.14	0.817	1.20
CS-19	0.699	1.12	0.682
CS-20	1.28	1.34	1.08

Table 10. C-myc, c-Ha-ras, and c-fos:TK ratios from slot-blot analyses of total cellular RNA from MFHs. also shown are TK:actin ratios which were used as molecular measures of cell cycle.

MFH SAMPLE	c-myc TK	c-Ha-ras TK	c-fos TK	TK actin
MT-1	0.455	0.686	0.419	0.501
MT-2	1.24	0.412	1.27	0.560
MT-3	1.14	0.465	2.54	2.03
MT-4	1.00	0.184	1.15	1.01
MT-5	0.870	0.781	0.987	0.636
MT-6	1.31	0.474	0.862	2.19
MT-7	0.690	0.412	0.953	0.651
MT-8	1.68	1.05	0.520	2.35
MT-9	0.935	0.360	0.417	0.602
MT-10	1.00	1.25	0.458	0.393
MT-11	1.09	3.87	0.556	1.31
MT-12	1.21	0.346	0.968	1.29
MT-13	1.26	0.370	0.472	1.46
MT-14	1.04	3.40	0.855	1.10
MT-15	1.15	0.258	0.908	1.51
MT-16	1.92	0.198	0.899	1.63
MT-17	1.97	0.498	0.895	2.13
MT-18	1.96	0.215	0.955	3.69
MT-19	1.05	0.145	1.25	1.30
MT-20	1.93	1.27	1.04	1.33
MT-21	1.14	0.896	1.30	1.06
MT-22	1.96	1.20	1.04	1.71
MT-23	1.10	0.987	1.30	1.03

Table 11. C-sis, v-erb-B-1, and v-src: actin ratios from slot-blot analyses of total cellular RNA from MFHs.

MFH SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
MT-1	1.25	0.113	0.283
MT-2	1.33	0.697	1.03
MT-3	1.65	1.27	0.616
MT-4	1.39	1.13	0.802
MT-5	1.30	1.38	1.94
MT-6	2.83	0.787	0.292
MT-7	1.54	0.680	1.70
MT-8	2.31	0.689	0.507
MT-9	1.14	1.35	0.992
MT-10	0.810	1.49	1.92
MT-11	2.29	1.02	2.92
MT-12	2.63	1.63	1.49
MT-13	2.19	1.50	1.71
MT-14	2.02	1.64	1.05
MT-15	1.56	1.17	0.790
MT-16	1.89	0.801	0.480
MT-17	34.0 **	0.832	2.32
MT-18	2.30	1.16	0.586
MT-19	1.87	0.558	1.04
MT-20	1.31	0.942	1.01
MT-21	1.29	1.09	1.01
MT-22	1.03	1.05	0.990
MT-23	1.43	1.05	1.01

** Value was determined from titration of RNA, since signal intensity was outside the linear range for this method.

Table 12. C-myc, c-Ha-ras, and c-fos: actin ratios from slot-blot analyses of genomic DNA from MFHs. Also shown are TK:actin ratios.

MFH SAMPLE	c-myc actin	c-Ha-ras actin	c-fos actin	TK actin
MT-1	0.833	1.26	0.991	0.800
MT-2	1.06	0.638	0.938	0.964
MT-3	1.05	0.956	0.815	1.05
MT-4	1.21	0.897	1.25	1.06
MT-5	1.18	1.31	1.07	0.919
MT-6	1.96	1.24	0.812	0.988
MT-7	1.17	1.06	1.04	0.891
MT-8	1.13	0.952	1.15	0.980
MT-9	1.26	0.916	1.32	1.27
MT-10	1.08	1.39	0.732	1.07
MT-11	1.14	0.834	0.908	1.01
MT-12	1.39	1.27	1.22	1.24
MT-13	1.19	1.15	0.998	1.14
MT-14	1.11	1.09	1.19	1.28
MT-15	1.21	1.27	1.09	1.18
MT-16	1.81	1.21	1.30	1.16
MT-17	2.25	1.20	1.26	1.21
MT-18	2.41	1.03	1.17	1.19
MT-19	1.01	1.11	1.27	1.20
MT-20	2.72	1.19	1.02	1.09
MT-21	1.25	1.28	1.20	1.19
MT-22	2.69	1.33	1.13	1.14
MT-23	1.45	1.31	1.29	0.960

Table 13. C-sis, v-erb-B-1, and v-src: actin ratios from slot-blot analyses of genomic DNA from MFHs.

MFH SAMPLE	c-sis actin	v-erb-B-1 actin	v-src actin
MT-1	1.15	1.37	0.946
MT-2	1.22	0.851	0.784
MT-3	0.759	1.13	0.947
MT-4	1.27	0.943	0.860
MT-5	1.17	1.01	1.02
MT-6	1.40	0.946	1.07
MT-7	0.868	0.978	1.08
MT-8	1.02	0.987	0.674
MT-9	1.35	0.976	1.03
MT-10	0.830	1.41	1.36
MT-11	1.34	0.891	0.777
MT-12	0.789	1.29	1.28
MT-13	1.17	1.28	1.14
MT-14	1.30	1.23	1.24
MT-15	1.13	1.36	1.17
MT-16	1.20	0.881	1.03
MT-17	1.22	1.24	1.05
MT-18	1.10	1.11	1.08
MT-19	1.30	1.24	1.35
MT-20	1.25	1.16	1.27
MT-21	1.33	1.22	1.27
MT-22	1.28	1.28	1.24
MT-23	1.32	1.15	1.26

(table 14). Mean TK:actin ratios are 1.1 in bone marrow, 1.1 in chondrosarcomas, and 1.5 in MFHs (figures 12 and 13). C-myc:TK ratios in bone marrows, chondrosarcomas, and MFHs were 0.9, 1.2, and 1.3 respectively. There are no significant differences between these groups for levels of c-myc transcript ($p>0.05$). C-Ha-ras and c-fos transcript levels among bone marrows, chondrosarcomas, and MFHs range from 0.8 to 1.1 and are not significantly different ($p>0.05$).

C-sis, v-erb-B-1, and v-src gene transcript levels are detectable in muscle, giving mean gene:actin values ranging from 0.9 to 1.0 (table 15). C-sis:actin ratios in bone marrows, chondrosarcomas and MFHs are 1.1, 1.6 and 3.1 respectively. There are significantly higher transcript levels of c-sis in MFHs compared to the other 3 groups ($p<0.05$) (figure 14). V-erb-B-1 and v-src transcript levels in bone marrows, chondrosarcomas and MFHs range from 0.9 to 1.2 and show no significant differences between any of the groups for transcript levels of these two genes ($p >0.05$).

DNA Slot-Blot Results and Determination of C-myc Gene Copy Number

Table 14. RNA quantitation of cell-cycle dependent genes as determined by slot-blot analysis. *

	MUSCLE	BONE MARROW	CS	MFH	
TK:ACTIN	ND	1.1±0.1	1.1±0.7	1.5±0.8	
C-MYC:TK	ND	0.9±0.2	1.2±0.7	1.3±0.4	**
C-HA-RAS:TK	ND	1.0±0.2	0.8±0.5	0.9±0.9	
C-FOS:TK	ND	1.0±0.1	1.1±0.4	1.0±0.4	
TOTAL CASES	9	6	20	23	

* Values shown are mean gene:actin and gene:TK ratios

** 17 MFHs with single copy **c-myc** had a mean **myc**:TK ratio of 1.0±0.2. Six MFHs with 2 or greater copies of **myc** had a mean **myc**:TK ratio of 2.0±0.1.

ND Not detectable

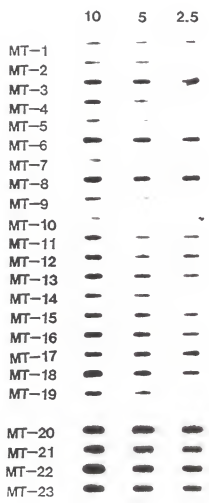


Figure 12. Slot-blot of total cellular RNA from MFHs hybridized with the TK probe. Quantities of 10, 5, and 2.5 ug of RNA were slot-blotted onto nitro-plus 2000, and hybridized with the TK probe (3.0 x 10 E6 cpm/ 10 E8 cpm/ug).

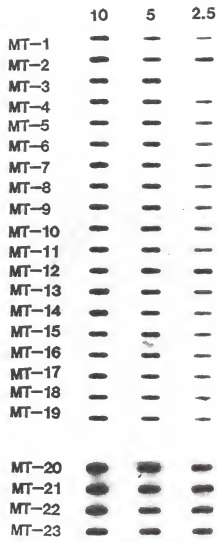


Figure 13. Slot-blot of total cellular RNA from MFHs hybridized with the actin probe. Quantities of 10, 5, and 2.5 ug of RNA were slot-blotted onto nitro-plus 2000, and hybridized with the actin probe (3.0 x 10 E6 cpm/ 10 E8 cpm/ug).

Table 15. RNA quantitation of non cell-cycle dependent genes as determined by slot-blot analysis. *

	MUSCLE	BONE MARROW	CS	MFH
C-SIS:ACTIN	1.0±0.1	1.1±0.1	1.6±1.5	3.1±6.8
V-ERB-B-1: ACTIN	0.9±0.1	1.2±0.2	1.1±0.7	1.0±0.4
V-SRC:ACTIN	1.0±0.1	1.1±0.1	0.9±0.2	1.2±0.7
TOTAL CASES	9	6	20	23

* Values shown are mean gene:actin ratios

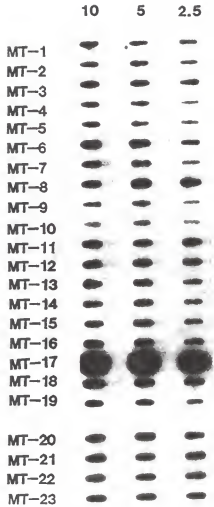


Figure 14. Slot-blot of total cellular RNA from MFHs hybridized with the c-sis probe. Quantities of 10, 5, and 2.5 ug of RNA were slot-blotted onto nitro-plus 2000, and hybridized with the c-sis probe (3.0 x 10 E6 cpm/ 10 E8 cpm/ug).

Tables 16 and 17 indicate the gene copy numbers for TK, actin, and the six genes evaluated in this study as determined by DNA slot-blot quantitation. **c-myc**:actin ratios for all 9 muscle, 6 bone marrow and 20 chondrosarcoma tissues show **c-myc** to be a single copy gene. The same is true for 17 of the MFHs, while 6 of these tumors have 2 or more copies of **c-myc**. **C-Ha-ras**, **c-fos**, **c-sis**, **v-erb-B-1**, and **v-src**:actin values indicate that these genes are single copy in all normal and neoplastic tissue groups.

The 6 MFHs with 2 or greater copies of **c-myc** are samples MT-8, MT-16, MT-17, MT-18, MT-20,and MT-22. When RNA values for this subset of MFHs were examined, it was found that these 6 MFHs have increased **c-myc** transcript levels as well (figure 15). Therefore, 17 of the MFHs have a mean c-myc:TK ratio of 1.0 which is not significantly different than that of chondrosarcomas (1.2), or bone marrow tissues (0.9). The 6 MFHs with 2 or greater copies of **myc** have a mean **myc**:TK ratio of 2.0 which is significantly higher than the other groups.

These 6 MFHs also have higher frequencies of cell division as measured by TK:actin ratios. Statistical evaluation of the relationship between **c-myc** gene copy number and cell division was performed using chi square analysis.

Table 16. DNA quantitation of cell-cycle dependent genes as determined by slot-blot analysis. *

	MUSCLE	BONE MARROW	CS	MFH
TK:ACTIN	0.9±0.1	1.0±0.2	1.0±0.1	1.1±0.1
C-MYC:ACTIN	1.1±0.1	0.9±0.2	1.0±0.1	1.5±0.6**
C-HA-RAS: ACTIN	1.1±0.1	0.9±0.1	0.9±0.2	1.1±0.2
C-FOS:ACTIN	0.9±0.1	1.0±0.2	1.0±0.2	1.1±0.2
TOTAL CASES	9	6	20	23

* Values shown are mean gene:actin ratios

** 17 MFHs with single copy **c-myc** had a mean **myc**:actin ratio of 1.2±0.1. Six MFHs with 2 or greater copies of **myc** had a mean **myc**:actin ratio of 2.3±0.4.

Table 17. DNA quantitation of non-cell-cycle dependent genes as determined by slot-blot analysis. *

	MUSCLE	BONE MARROW	CS	MFH
C-SIS:ACTIN	0.9±0.1	1.0±0.1	1.2±0.2	1.2±0.2
V-ERB-B-1: ACTIN	1.0±0.1	1.0±0.2	1.0±0.2	1.1±0.2
V-SRC:ACTIN	0.9±0.3	0.9±0.1	1.2±0.2	1.1±0.2
TOTAL CASES	9	6	20	23

* Values shown are mean gene:actin ratios

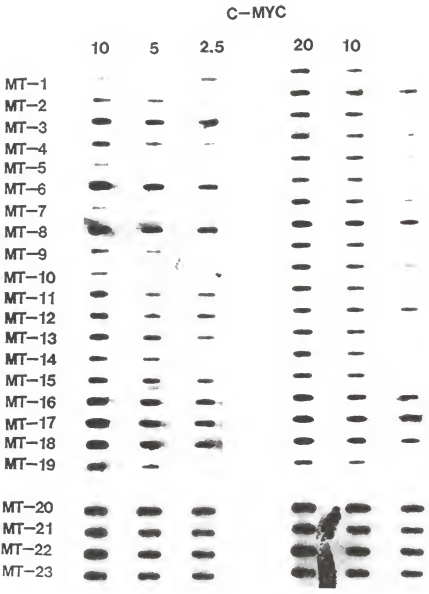


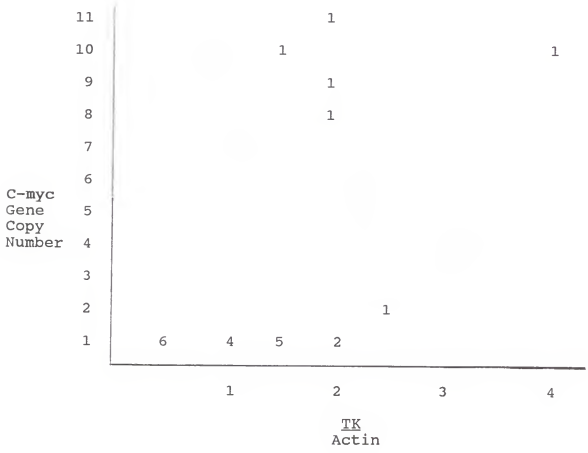
Figure 15. Slot-blot of total cellular RNA and genomic DNA from MFHs hybridized with the **c-myc** probe. Quantities of 10, 5, and 2.5 ug of RNA and 20, 10, and 5 ug of DNA were slot-blotted onto nitro-plus 2000, and hybridized with the **c-myc** probe (3.0 x 10 E6 cpm/ 10 E8 cpm/ug).

The results indicated a positive correlation between **c-myc** gene copy number and cell division as represented by TK:actin ratios ($p<0.05$) (table 18).

The slot-blotting technique used in these studies was primarily a technique for screening total cellular RNA and genomic DNA from tumors and normal tissues for proto-oncogene expression. A restricted range of linearity for hybridization signal intensities was one technical limitation of this system, therefore limiting its reliability as a quantitative tool when signal intensities exceeded or fell short of the linear range. For example, DNA copy numbers greater than 2 could not be assessed in 6 MFHs. Another technical limitation of the slot-blot is that it cannot be ascertained what size transcript the probe hybridized to. In these analyses, all probes were confirmed for correct specificity by restriction digests and Southern blot analysis prior to use in slot-blot hybridizations.

The beta-actin control was the most validating factor for the slot-blotting assay used in this study. Hybridization with this gene is an internal control for both RNA and DNA slot-blots. As shown in these results, actin values show little variability between samples on both RNA

Table 18. RNA TK:actin ratios and **c-myc** gene copy numbers for MFHs. Chi square analysis showed a positive correlation between **c-myc** gene copy number and cell division as measured by TK:actin ratios ($p \leq 0.05$). The cut off point was 2 for **c-myc** gene copy number and for TK:actin values.



and DNA slot-blots. This serves as a control for a single copy, constitutively expressed gene for RNA and DNA analyses. It also demonstrates consistency of quantity and quality of RNA.

Since quantitative signal intensity limitations of the slot-blotting assay do not allow determination of gene copy numbers greater than two, c-myc copy numbers for the 6 MFHs with 2 or greater copies of the gene were further evaluated using Southern blot/ DNA dilutional analysis.

Tumor DNA samples from 6 MFHs (mt-8, mt-16, mt-17, mt-18, mt-20, and mt-22) and from normal muscle tissues were prepared as described above. Aliquots of 10, 5, 2, and 1 ug of tumor DNA and 10 ug of normal muscle DNA were analyzed using Southern blot hybridization with the p GEM H MYC probe (table 1). This probe hybridizes to a 9.6 kb fragment of Hind III restricted genomic DNA. Band sizes were determined by comparison to lambda DNA marker bands produced by restriction with Hind III (figure 16). **C-myc** gene copy numbers were determined by laser densitometry (transmittance). Values for tumor DNA are normalized to those of muscle DNA which are controls for single copy c-myc. These 6 MFHs were found to have **c-myc** gene copy numbers of (A-F) 8.7, 8.4, 9.6, 9.9, 10.6, and 2.2, which corresponded

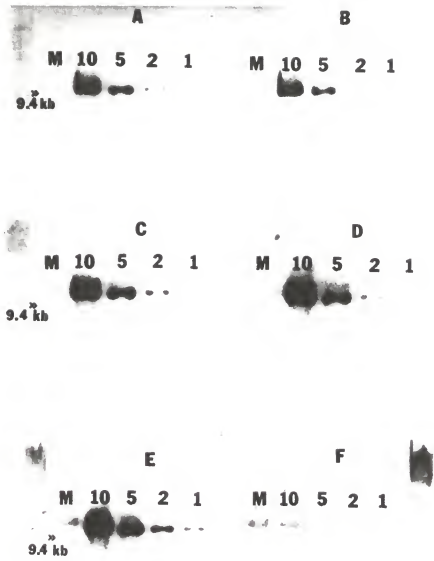


Figure 16. Southern blot/DNA dilutional analysis of genomic DNA from MFHs with 2 or greater copies of **c-myc**. Tumor DNA samples of 10, 5, 2, and 1 ug (labeled 10, 5, 2 and 1) and muscle DNA samples of 10 ug (labeled M) were digested with Hind III, electrophoresed through 0.8 percent agarose gels, blotted, and hybridized with p GEM H MYC. **C-myc** gene copy numbers were calculated to be (A-F) 8.7, 8.4, 9.6, 9.9, 10.6 and 2.2 respectively. Samples A,B,C,D,E, and F correspond to samples MT-16, MT-17, MT-18, MT-20, MT-22, and MT-8 respectively as shown in figure 15.

to MT-16, MT-17, MT-18, MT-20, MT-22, and MT-8 respectively. Thus, five of the MFHs with increased transcript levels of c-**myc** have between 8 and 11 copies of the **c-myc** gene, while a sixth has 2 copies.

Regions Contained in the C-Myc Amplicon

The Southern blots from the dilutional analysis of **c-myc** copy number described above were rehybridized with the **c-myc**, p 380-8A, H25-8A, and HT .96 probes to further examine the copy number of the **c-myc** gene and surrounding regions in the 6 MFHs with **c-myc** amplification. This was done in order to determine whether the entire **c-myc** gene was amplified, and to determine if the promoter region was contained in the amplicons. Copy numbers for the different areas of the **c-myc** gene and surrounding areas were determined by laser densitometry as described above for Southern blot dilutional analysis of **myc** gene copy number.

Hybridization with the **c-myc** probe from Oncor which is specific for all three **myc** exons, indicates that this region is amplified approximately 10 times in mt-16, mt-17, mt-18, mt-20, and mt-22, while mt-8 has 2 copies (table 19). The same is true for the p GEM H MYC probe (discussed above)

Table 19. Copy numbers of 5' and 3' regions of the c-myc gene and flanking regions in mt-8, mt-16, mt-17, mt-18, mt-20, and mt-22 as determined by Southern blot analyses of genomic DNA digested with Hind III and hybridized with the c-myc, p GEM H MYC, p 380-8A, H25-3.8, and HT 0.96 probes. *

	Probe				
	C-myc	pGEM H MYC	p380-8A	H25-3.8	HT 0.96
Size of hybridized fragment (kb)	9.6	9.6	6.8	9.4	3.4
Tumor					
MT-8	2	2	2	1	1
MT-16	9	9	9	1	1
MT-17	9	8	9	1	1
MT-18	10	10	10	1	1
MT-20	10	10	10	1	1
MT-22	10	11	11	1	1
MUSCLE	1	1	1	1	1

* Copy numbers were determined by laser densitometry. Values were normalized to that of muscle which has a **c-myc** gene copy number of 1.

which was specific for the 3' end of the gene. These probes were specific for overlapping regions, and both were used for confirmatory purposes.

The p 380-8A probe which is specific for a region approximately 50 kb upstream from the **c-myc** promoter indicates an amplification of approximately 10 fold in mt-16, mt-17, mt-18, mt-20, and mt-22, while mt-8 has 2 copies of this region (table 19). Analyses with the thyroglobulin and carbonic anhydrase probes, show single copy genes (table 19). These results indicate that the amplfied regions of **myc** (8q 24) in MFHs are very large, and contain all three exons as well as regulatory regions. The amplicons do not extend as far 5' as the carbonic anhydrase gene (8q 22), or as far 3' as the q terminal region of chromosome 8, where the thyroglobulin gene is located.

Chromatin Structure Analysis

C-Myc Gene Copy Number and Transcript Levels in P3C, UR HCL 1, HFF and ST 486 Cell Lines

C-myc gene copy numbers were determined for the P3C and UR HCL 1 cell lines. Twenty, 10, 5, and 2 ug of genomic DNA

from these cell lines were prepared as previously described, and analyzed using the pGEM H MYC probe and conditions established for Southern hybridizations. Gene copy number was determined by laser densitometry and normalized to muscle DNA. The P3C cell line was found to contain approximately 10 copies of the c-myc gene, while the UR HCL 1 cell line has a single copy c-myc gene (figure 17).

Five and 10 ug aliquots of total cellular RNA from the P3C, UR HCL 1, HFF, and ST 486 cell lines were evaluated for relative c-myc transcript levels using northern blot hybridization methods and the p GEM H MYC probe. The blot was rehybridized with the human beta actin gene probe as a control for RNA quantitation. P3C cells clearly showed increased levels of c-myc transcript relative to the other cell lines (figure 18).

Locations of DNAse I Sites in P3C, UR HCL 1, HFF, and St 486 Cell Lines

Data generated thusfar support the hypothesis that increases in c-myc transcript are due to gene amplification. The possibility that other regulatory changes may contribute to this as well cannot be ruled out. Therefore, potential differences in chromatin structure were evaluated between

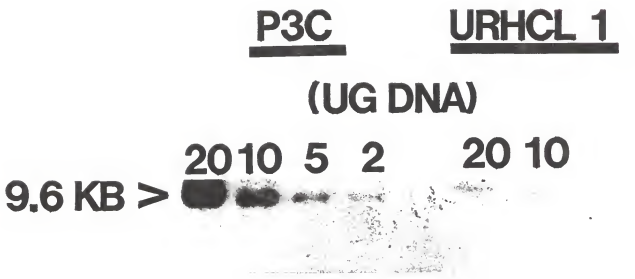


Figure 17. Southern blot analysis of **c-myc** DNA copy number in UR HCL 1 and P3C cell lines. Twenty, 10, 5, and 2 ug of DNA from each cell line were restricted with Hind III, electrophoresed through 0.8 percent agarose gels and hybridized with the pGEM H MYC probe (See materials and methods). Band size was determined by lambda DNA marker bands produced by restriction with Hind III.

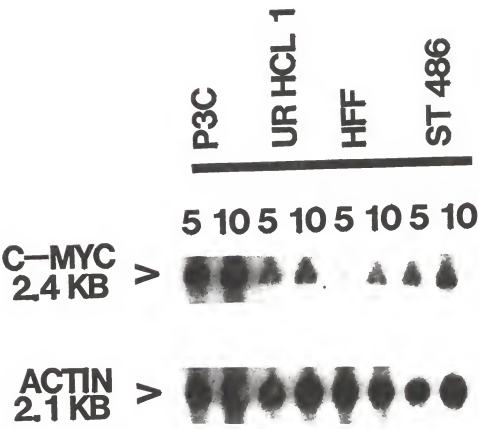


Figure 18. Northern blot analysis of total cellular RNA from UR HCL 1, P3C, HFF, and ST 486 cell lines. Ten and 5 ug aliquots of RNA from each cell line were electrophoresed through 1.2 percent formaldehyde agarose gels, blotted, and hybridized with the pGEM H MYC and beta actin probes (See materials and methods). Message sizes were determined by an RNA ladder.

amplified and single copy c-myc genes in MFH cell lines. Locations of c-myc DNAse I hypersensitve sites were determined for P3C, UR HCL 1, HFF, and ST 486 cell lines. Initially, mapping of these sites was done from a 3' direction using previously described methods. Various concentrations of DNAse I were used for each cell line to determine an optimal range of concentrations (i.e. ones which yielded optimum visualization of DNAse I generated bands). Figure 19 shows Southern blot analysis of DNAs from DNAse I treated nuclei from the UR HCL 1 cell line. This figure demonstrates the extent of digestion with varying concentrations of DNAse I.

Genomic DNA was isolated from DNAse I treated nuclei as previously described. Fifteen ug of DNA from the UR HCL 1, HFF, and ST 486 cell lines and 7 ug of DNA from P3C DNAse I treated nuclei were restricted with ECO R1, and compared for locations of DNAse I hypersensitive sites by Southern blot hybridization with the pmc 41 probe (figure 20).

The optimum DNAse I concentrations for the P3C, UR HCL-1, and HFF lines were 0.2, 0.5, and 1.0 ug/ml DNAse I, while those for ST 486 were 0.1, 0.2, and 0.5 ug/ml DNAse I. The controls shown for each cell line were 0 ug/ml DNAse I at 37 C degrees (lane marked 0). Locations of DNAse I hypersensitive sites were determined by comparison to lambda

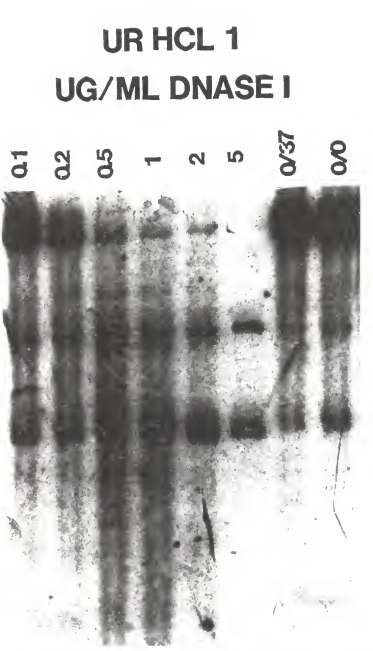


Figure 19. Southern blot of UR HCL-1 genomic DNA from DNAse I treated nuclei. After treatment of nuclei with various concentrations of DNAse I (L-R; 0.1, 0.2, 0.5, 1, 2, 5, ug/ml, controls consisted of 0 ug/ml DNAse I at both 0 and 37 C degrees), genomic DNA was isolated , restricted with Eco R I, electrophoresed through 0.8 percent agarose gels, and hybridized with the pmc 41 probe. Samples 0.2, 0.5, and 1 ug/ml DNAse I gave optimum band visualization and were used in a composite Southern blot shown in figure 20.

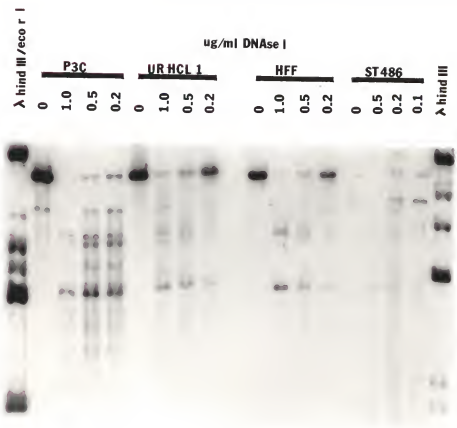


Figure 20. Southern blot of P3C, UR HCL 1, HFF, and ST 486 genomic DNA from DNAse I treated nuclei. The concentrations of DNAse I shown for each cell line were the ones which gave optimal visualization of bands in the initial analyses. The genomic DNA from DNAse I treated nuclei was restricted with Eco R I, electrophoresed through 0.8 percent agarose gels, blotted, and hybridized with the pmc 41 probe. Controls shown for each cell line were 0 ug/ml DNAse at 37 C degrees.

DNA digested with either Hind III (right side of blot), or both Eco R1 and Hind II together (left side of blot).

Controls at 0 ug/ml DNAse I at 0 and 37 C degrees demonstrated that DNAse I generated bands are real, and not due to endogenous nuclease activity. There is a band seen in the control lane for the P3C cell line. Although this band may be generated by endogenous nucleases, it ran differently than the DNAse I band of similar size, and was not relevant to these analyses.

Locations of DNAse I hypersensitive sites for the c-myc gene in each of the cell lines studied are shown in figure 21. It was found that five DNAse I hypersensitive sites at identical locations are present for UR HCL 1 and the normal human fibroblast line HFF (sites 1,2,3,4,6, figure 21). One site is located 5' to the first exon and 5' of promoter P0 (site 1). Two sites are located 5' of the first exon and 3' of the P0 promoter region (sites 2,3). Another site is located in the 3' region of the first exon near a PVU II site (site 4), and a fifth site was found to be 5' of exon 2 (site 6). The amplified c-myc gene in the P3C cell line also had four of these sites (1,3,4,6), however a site near the P0 promoter region is not present (site 2), and a new site in the 5' region of the first intron is seen (site 5). Each of

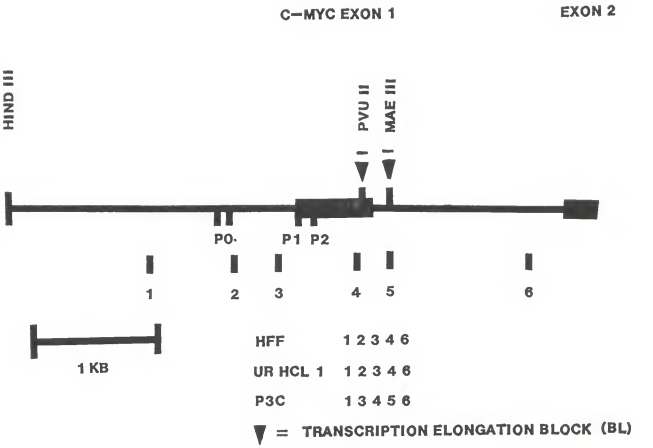


Figure 21. Locations of DNAse I hypersensitive sites in the c-myc gene for each of the cell lines HFF, P3C, and UR HCL-1. Also shown are promoter regions P0, P1, and P2.

the DNAse I bands for P3C cells were similar in intensity. This suggests that most if not all of the copies of c-myc have these changes in chromatin structure. Three DNAse I hypersensitive sites were observed for the ST 486 cell line. These were in identical regions as those previously reported by Siebenlist et al. (156) in Burkitt lymphoma BL 31 cells.

Chromatin Structure of the C-myc Gene During the G0/G1 Transition in the HFF Normal Human Fibroblast Cell Line

These data show that increases in transcript and changes in DNAse I hypersensitive sites accompany c-myc gene amplification in P3C cells. It was of interest to determine if changes in DNAse I hypersensitive sites are seen in normal cells during periods when peak levles of c-myc transcript are produced. Increased levels of c-myc transcript production have been observed during the G0/G1 transition in quiescent fibroblasts after serum addition. Quiescent HFF cells were evaluated at G0 , 0.5, 1, 2, and 3 hours after serum release, and during log phase growth. Locations of DNAse I hypersensitive sites in these cells were mapped from the 3' direction using Southern hybridization with the pmc 41 probe.

Total cellular RNA from the cells at each of these time points was evaluated by Northern blot analysis for transcript levels of c-myc and TK to ascertain that the desired phases of the cell cycle were represented (figure 22). Hybridization with the c-myc probe demonstrates a profile of transcript levels which starts out at a basal level during G0, peaks 1 hr after serum release, then returns to levels comparable to those of G0 during log phase growth. Levels of TK at the various time points indicate that transcript levels are highest during log phase, and lowest during G0. Hybridization with actin demonstrates consistent actin transcript levels and RNA quantitation. These results are expected for transcript levels of these genes during the cell cycle.

Fifteen ug of genomic DNA isolated from DNAse I treated nuclei at each time point discussed above were restricted with ECO R1 and analyzed for Locations of DNAse I hypersensitive sites as previously described (figure 23). Mapping of sites was accomplished by band size comparisons to those generated by digestion of lambda DNA with Eco R 1 and Hind III. Locations of c-myc DNAse I hypersensitive sites for HFF at each time point examined were found to be identical to those previously described for HFF (sites

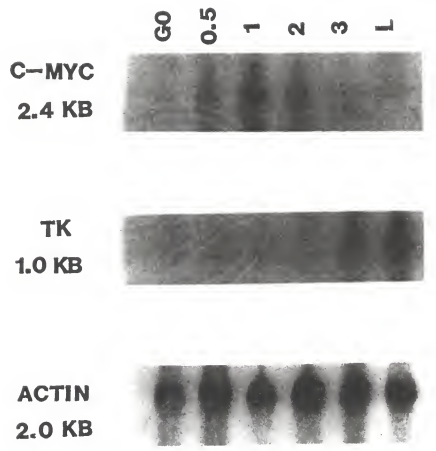


Figure 22. Northern blot analysis of total cellular RNA from the HFF normal human fibroblast cell line. HFF cells were made quiescent (G0) in MEM containing 0.1 percent fetal bovine serum (37 C degrees, 3 days), then released by addition of MEM supplemented with 10 percent fetal bovine serum. RNA samples were evaluated for levels of c-myc, TK and actin transcript at G0, 0.5, 1, 2, and 3 hours after serum release, and during log phase growth (L) (see materials and methods).

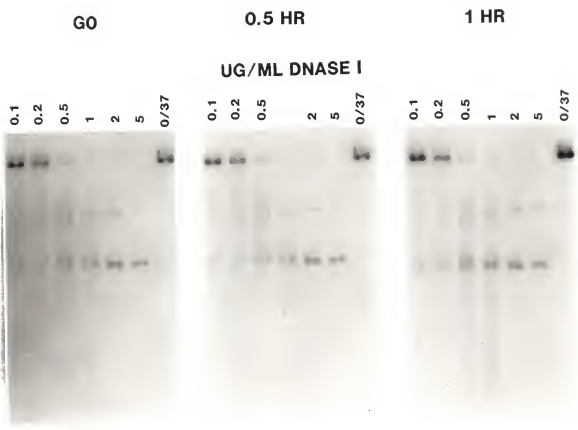


Figure 23. Southern blot analysis of genomic DNA from DNAse I treated nuclei of HFF normal human fibroblast cells. Nuclei isolated from HFF cells in G0, 0.5, 1, 2, and 3 hours after serum release, and during log phase growth (L) were treated with various concentrations of DNAse I (L-R; 0.1, 0.5, 1, 2, 5, and 0 ug/ml (37 C degrees)). Genomic DNA was isolated, restricted with Eco R 1, electrophoresed through 0.8 percent agarose gels, blotted, and hybridized with the pmc 41 c-myc probe (See materials and methods). Sizes of bands were determined by comparison to lambda DNA digested with Eco R 1 and Hind III.

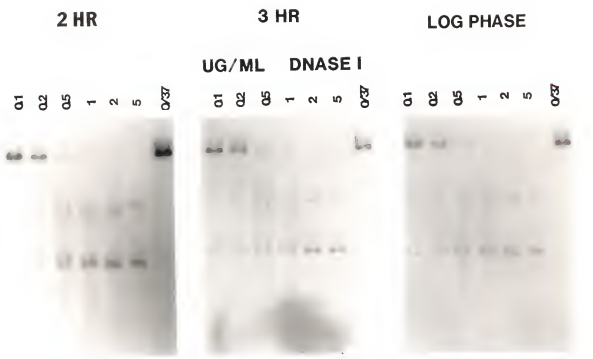


Figure 23. contd.

1,2,3,4,6) in figure 21. Despite a peak of **myc** transcript production 1 hr after serum release, no changes were observed in the locations of these sites during the transition of HFF fibroblasts from G0 to G1, or between any time points after serum release.

Fine Mapping Analysis of DNAse I Hypersensitive Sites in P3C Cells From a 5' Direction

Fine mapping of P3C c-**myc** DNAse I hypersensitive sites (shown in figure 21) in the exon 1/ intron 1 region was done from the 5' direction to more precisely determine their locations (particularly site 5) relative to known transcription elongation block sites. Five ug of P3C and 15 ug of HFF DNA from DNAse I treated nuclei were restricted with Sca I. These, and P3C marker DNAs restricted with Sca I/Mae III, Sca I/Bsm I, and Sca I/Pvu II were analyzed by Southern blot hybridization using the Sca I/Xho I probe (see materials and methods). The Southern blot from fine mapping analysis of c-**myc** DNAse I hypersensitive sites in the 3' region of exon 1, and in intron 1 is shown in figure 24. The resolution of bands on this blot was found to be at least 25 base pairs. This is demonstrated by the easy resolution of bands generated by digestion with Sca I/Mae III (970 bases)

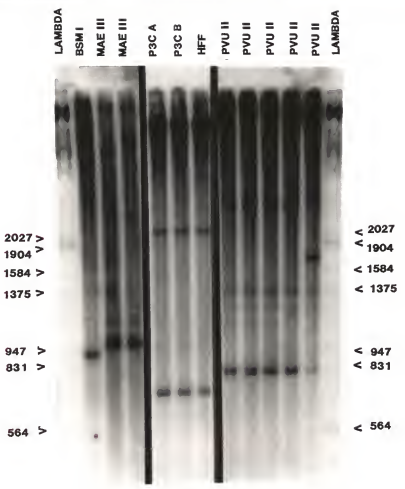


Figure 24. Southern blot of P3C and HFF DNAse I treated DNAs used in fine mapping of DNAse I hypersensitive sites in the exon 1/ intron 1 region. Also shown are P3C genomic DNA marker fragments; ScaI/Bsm I, Sca I/ Mae III, and ScaI/Pvu II. P3C A and P3C B were treated with 0.2 and 0.5 ug/ml DNAse I respectively (from figure 20). The HFF sample (from figure 20) was treated with 0.5 ug/ml DNAse I. The blot was hybridized with the 355 b.p. Sca I/ Xho I fragment (2.0 X 10 E7 cpm/ml/ 10 E8 cpm/ug) (see materials and methods). Also shown are lambda DNA markers produced by digestion with Eco R I and Hind III.

and Sca I/ Bsm I (920 bases) which differ by 50 base pairs and are 0.5 cm apart on the blot. Lambda DNA digested with Eco R I/Hind III was run on either side of the gel as size markers, and to assure the gel ran evenly. Sizes of these marker bands are also shown in figure 24. Two samples of DNAse I treated DNAs from P3C cells (0.2 and 0.5 ug/ml, A and B respectively) were run because both produced optimal visualization of DNAse I generated bands, as did sample 0.5 ug/ml for HFF cells. Bands corresponding to sites 4 and 6 (figure 25) were observed in both the P3C and HFF cell lines, and map to distances of 760 and 2025 base pairs 3' of the Sca I site respectively. Site 5, which is present exclusively in the P3C cell line, maps to a distance of 960 base pairs downstream of the Sca I site. As a result of these analyses, site 4 can be placed approximately 45 base pairs 5' of the Pvu II site in one region known to be a transcription attenuation site. Site 5 can be placed in a region 3' of exon 1, approximately 10 base pairs 5' of an Mae III site. This site is located in a region also known to contain a transcription elongation block, which extends 15 base pairs 5' and 5 base pairs 3' of the Mae III site. Site 6 was found to be approximately 465 bases 5' of exon 2 in the same region as an S1 nuclease sensitive site described by Grosso and Pitot (73).

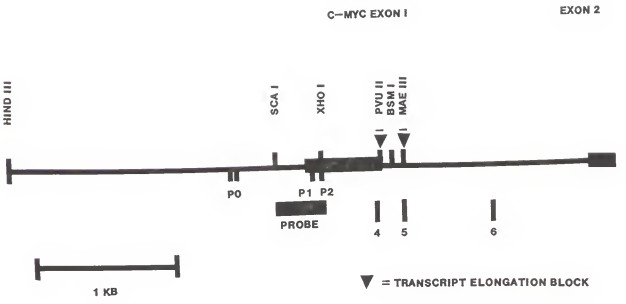
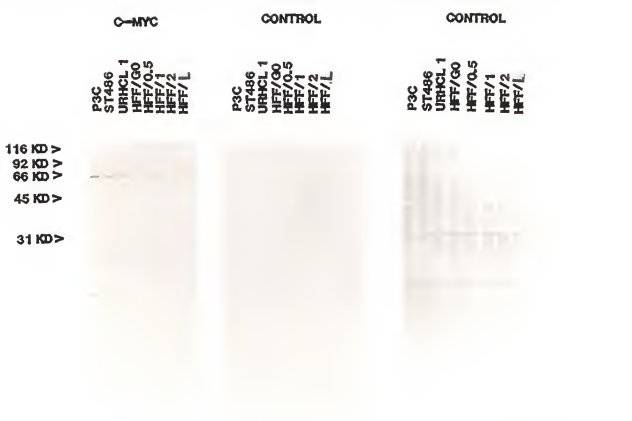


Figure 25. Locations of c-myc DNAse I hypersensitive sites 4, 5, and 6 for P3C and HFF cell lines as determined from fine mapping analysis from the 5' direction.

C-myc Protein Levels in the P3C, UR HCL 1, HFF, and ST 486 Cell Lines

A comparison of **c-myc** protein levels was made between cells with amplified (P3C) and single copy (UR HCL 1) **c-myc** genes, cells in which **c-myc** is thought to be an oncogene (ST 486), and normal fibroblasts during peak levels of **c-myc** transcript production. This was done to determine if the increased levels of **c-myc** transcript seen in P3C cells were translated. Twenty ug of protein from P3C, UR HCL 1, ST 486 and HFF cells (G0, 0.5, 1, and 2 hours after serum release, and during log phase growth), were evaluated for relative **c-myc** protein levels using PAGE and Western blotting techniques as previously described.

The **c-myc** monoclonal antibody (HL-40) bound to a 65 kd protein band (132) as determined by molecular weight markers run on the gel. The control panel, incubated with anti-met 72 (72/K 88.151.G127), showed no non-specific binding, and staining with india ink indicated that protein quantitation was consistent between samples (figure 26). Relative levels of **c-myc** protein were determined by reflectance densitomety, and values were normalized to those of fibroblasts in G0 (figure 26). Fibroblasts 0.5 and 2 hours after serum



P3C	ST 486	UR HCL 1	HFF/G0	HFF/0.5	HFF/1	HFF/2	HFF/L
6	5	2	1	1	2	1	1

Figure 26. Western blot showing relative levels of **c-myc** protein in P3C, UR HCL 1, ST 486, and HFF (G0, 0.5, 1.0, 2.0 hr after serum release, and during log phase growth (L)) cell lines. Twenty ug of protein were separated by size using PAGE, and electroblotted onto nitrocellulose. The panels were (L-R) incubated with the HL-40 (anti **c-myc**) monoclonal antibody, the K 88.151.G127 (anti met 72) antibody (control for non-specific binding), and the third panel was stained with 0.1 percent india ink to control for protein quantitation. The **c-myc** monoclonal antibody bound to a 65 kd protein band as determined by molecular weight markers. Shown below the figure are the relative levels of **c-myc** protein as determined by reflectance densitometry. Values were normalized to **myc** protein levels in HFF cells at G0 which was given a value of 1.

release, and during log phase growth had the same amounts of c-**myc** protein as in G0. Protein levels in UR HCL 1 cells and fibroblasts 1 hr after serum release (presumably G0/G1 transition) were twice this level (2), while ST 486 and P3C cells had 5 and 6 times as much protein respectively.

CHAPTER 5
DISCUSSION

An amplified c-myc gene and increased levels of c-myc and c-sis transcript suggest an involvement of these genes in the pathogenesis and progression of MFHs. No increased transcript levels or amplified copy numbers of any of the proto-oncogenes were found in chondrosarcomas. MFHs are more malignant, have a higher fraction of dividing cells (39), and are potentially more genetically unstable. It is therefore not surprising that more proto-oncogene mutations would be observed in these tumors.

Muscle and bone marrow specimens were compared as examples of normal non-dividing and dividing mesenchymal tissues. Results presented here show that transcript levels of TK, c-myc, c-Ha-ras, and c-fos are undetectable in skeletal muscle tissues while levels of c-sis, v-erb-B-1, v-src and actin are present at detectable levels. These results are expected since normal skeletal muscle is a non-dividing tissue and c-myc, c-Ha-ras, c-fos, and TK are cell cycle dependent genes, while c-sis, v-erb-B-1, v-src and

actin are non-cell-cycle dependent. It has been reported that actin transcript levels vary during stages of the cell cycle (69). However, later studies with quiescent fibroblasts have shown that beta actin message levels were consistent during all phases of the cell cycle after serum release (175). These studies suggest that levels of actin transcript production during phases of the cell cycle may depend on cell type and culture conditions. Data reported here indicates that actin levels are consistent during the cell cycle in normal HFF fibroblasts. Therefore, actin was used in normalization of results for slot-blot and northern blot analyses.

These results for the **v-erb-B-1** and **v-src** genes are somewhat in agreement with those reported by Claycomb and Lanson (27) and Leibovitch et al. (107) which show that **c-myc**, **c-Ha-ras**, c-sis, **c-src**, and **v-erb-B-1** transcripts are present in skeletal muscle cells in culture while those of TK and **c-fos** are not.

Transcript levels of cell cycle dependent proto-oncogenes and TK as well as non-cell-cycle dependent proto-oncogenes and actin were found to be detectable in all bone marrow samples. It would be reasonable to suspect that all of these proto-oncogenes including cell-cycle dependent genes

would be expressed because bone marrow is a normally dividing tissue; this is what was observed. Detectable c-fos transcript levels have also been observed in normal bone marrow by Evinger-Hodges et al. (58).

The slot-blotting technique has advantages for an analysis of this nature. Due to the moderately degraded conditions of most RNA samples isolated from surgically obtained tumor and normal tissue specimens (as described in materials and methods), slot-blotting provides a workable alternative to northern blotting which is not possible with degraded RNA.

The most reliable information provided by the slot-blot assay is a relative comparison of proto-oncogene transcript levels between samples. This was accomplished by maintaining constant pre-hybridization and hybridization conditions, as well as film exposure times.

The slot-blot results presented here do not rule out a tumorigenic involvement of proto-oncogenes which do not have abnormal transcript levels or copy numbers. They do however, offer some clues as to which potential proto-oncogene activation mechanisms may be at work in the cases of the c-myc and c-sis genes in MFHs.

Several possible mechanisms have been described for the

activation of proto-oncogenes to oncogenes. It is possible that any of these; insertional mutagenesis, enhancer/promoter activity, amplification, gene rearrangements or point mutations could result in the loss of normal transcriptional constraints. It is reasonable to expect that increases in transcriptional levels would result from all of these mechanisms; including point mutations, if in regulatory rather than coding regions.

Single copy genes and insignificant differences in transcript levels between normal and tumor tissues were observed for c-Ha-ras, c-fos, v-erb-B-1, and v-src. Although no known mechanism of proto-oncogene activation is apparent (above), this does not exclude an involvement of these genes through point mutations at critical sites or some unknown activation mechanism. In the case of the **c-myc** gene in 6 MFHs, evidence presented here supports the concept of gene amplification as a mechanism of activiation of a proto-oncogene to an oncogene. Furthermore, TK:actin ratios indicate that **myc** gene amplification may be driving cell division in these tumors.

Increased transcript levels of **c-myc**, together with multiple copies of the gene, suggest that abnormal amounts of transcript are due at least in part to gene dosage effects.

Increases in transcript levels of c-sis were observed in MFHs; however, only single copies of the c-sis gene were seen. This suggests that increases in sis transcript levels are due to some undetermined mechanism other than gene amplification.

The myc gene product is postulated to be a double stranded DNA binding protein capable of participating in the regulation of cell division (17, 94, 95, 96). In vitro experiments suggest that c-myc genes are of a cell-cycle dependent nature in that levels of c-myc transcript like those of c-jun (143) and c-fos (120) increase during the G0/G1 transition and decrease to G0 levels during S-phase. These are unlike more "traditional" cell-cycle dependent genes such as histone H-2b and TK whose transcript levels peak during S phase (175).

Beta-actin is commonly used as a standard to normalize the expression of other genes because it is single copy, and constitutively expressed in most tissues. In these studies, it was appropriate to normalize to TK instead of actin for transcript levels of cell cycle dependent proto-oncogenes; c-myc, c-Ha-ras, and c-fos. Normalization to actin would not correct for the different frequencies of cell division seen in different tumors. Although the c-myc gene is not cell cycle dependent in the "traditional" sense, normal tissues or

tumors with a larger growth fraction would be expected to have increased **c-myc** transcript levels regardless of the factors driving cell division.

Southern blot analyses show that **c-myc** amplicons in MFHs (which contain approximately 10 copies) are very large and contain all three exons. Hybridization with the p380-8A probe (specific for a region approximately 50 kb upstream from the **c-myc** promoter) indicates that the **c-myc** promoter region is amplified as well. Use of this probe in similar analyses by Haluska and Croce (78) has shown this region to be co-amplified with the **c-myc** gene in COLO 320 (colon carcinoma cell line), but not with **c-myc** in HL-60 cells.

The measurements reported here for **c-myc** gene amplification are in accordance with other studies which have demonstrated **c-myc** gene amplification of large regions of DNA in other tumor systems (2). The question of why **c-myc** amplification occurs at all has been the focus of much speculation. In the case of Wilm's tumor for example, high levels of transcript have been observed from a single gene (2). If **c-myc** is a nuclear regulatory protein found in most normal cells then why would normal growth provide an ever increasing pressure for selection of cells with elevated levels of **c-myc** protein? The type of selective pressures

required to produce a cell with an amplified **c-myc** gene are unknown. It has been postulated by Alt (2) and others, that if **c-myc** genes could regulate expression of other genes, then maybe amplification is selecting for regulation of various growth regulatory genes.

The data reported here are consistent with the hypothesis that increases in **c-myc** transcript production are due to gene amplification. A hypothesis that changes in chromatin structure exist between amplified and single copy **c-myc** in MFH cell lines was tested as well. Studies with DNAse I demonstrated differences in chromatin structure between amplified and single copy **c-myc** genes in MFH cell lines. Changes which accompanied **c-myc** gene amplification include the disappearance of a DNAse I hypersensitive site 5' of exon 1, and the appearance of a new site in the first intron. The meaning of these data can be more fully realized when compared to those of Siebenlist and Leder (156), and Siebenlist and Kelly (155) (figure 27). These two studies reported that changes in chromatin structure accompanied **c-myc** structural mutations in Burkitt lymphoma (translocation) and HL-60 cells (amplification) (figure 27).

DNAse I hypersensitive sites 2 and 3 for HFF and UR HCL 1 cell lines, and site 3 in P3C cells were located in the

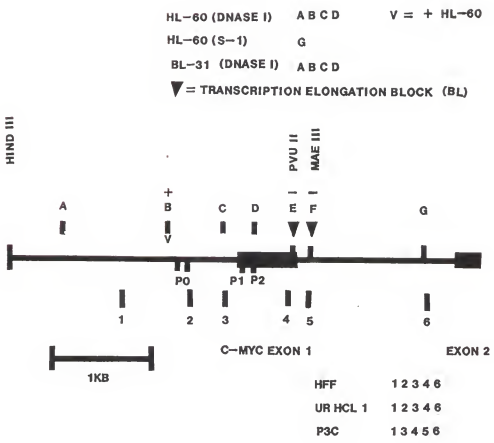


Figure 27. Summary of chromatin structure analyses previously described for the c-myc gene (letters) and those reported here (numerals). Sites A, B, C, and D are DNAse I hypersensitive sites found in both HL-60 cells (Siebenlist et al (155)), and Burkitt lymphoma (BL-31) cells (Siebenlist et al. (156)). Site B (indicated by open arrow) has been described by Siebenlist et al (155) to be involved in the maintainance of c-myc transcript production in HL-60 cells, and is therefore marked with a (+) symbol. Sites E and F (solid arows) represent transcription attenuation sites found in Burkitt lymphoma biopsies and cell lines and are marked by a (-) symbol (25, 199). Site G is an S-1 nuclease sensitive site described by Grosso and Pitot (73) in HL-60 cells.

same regions as sites B and C (C for P3C cells) in HL-60 and Burkitt lymphoma cells. Site 2, which is not seen in P3C cells, was located in the same region as site B in HL-60 cells. The disappearance of this site has been shown to accompany decreased **c-myc** transcript production in differentiating HL-60 cells post treatment with DMSO. Even though site 2 and site B map to different sides of the P0 promoter, they can be considered to be located in the same region because DNAse I hypersensitive sites may include 150-200 base pairs (about the size of a nucleosomal repeat). Site 4 in HFF, UR HCL 1, and P3C cells was located in a region which contains a PVU II site and a **c-myc** transcript elongation block in Burkitt lymphoma biopsies and cell lines (Site E, figure 27) (25). Site 5 was observed exclusively in P3C cells. This site mapped to a region in the first intron also known to contain a **c-myc** transcript elongation block in Burkitt lymphoma cell lines (Site F, figure 27) (199). Site 6 in the HFF, UR HCL 1, and P3C cell lines has not yet been found by DNAse I hypersensitive site analysis. However, an S-1 nuclease sensitive site in a similar region has been described by Grosso and Pitot (73).

The DNAse I sensitivity assay can provide general locations of DNAse I hypersensitive sites. Considering the potentially large areas of these "sites", resolution of this technique may be low. This may explain why site A in lymphocytes and site 1 in UR HCL 1, HFF, and P3C cells mapped to different locations (figure 27). Another possibility is that some DNAse I sites for a particular gene are cell-type specific, while others are shared between cell types.

Although a transcript attenuation site in the same area as sites E and F (figure 27) has been proposed for amplified c-**myc** in HL-60 cells, a precise location has not yet been described. The importance of fine mapping P3C c-**myc** DNAse I hypersensitive sites in this region can therefore be appreciated. Sites in the exon 1/intron 1 region were fine mapped from a 5' direction, and it was found that site 5 mapped to the same location as site F, which was one of the transcript attenuation sites previously described (figure 27).

Based on the changes in chromatin structure seen with the c-**myc** gene in P3C cells, one would expect to see decreased levels of **myc** transcript. This conflicts with what was observed with northern blot analysis. Therefore

potential changes in chromatin structure during c-myc upregulation in normal cells were studied. It has been shown that c-myc transcript levels peak during the G0/G1 transition when serum starved fibroblasts are released from their quiescent states (175). Although transcript levels peaked 1 hr after serum addition in HFF cells as shown by northern blot analysis, no differences in DNAse I sensitive sites were observed between fibroblasts in G0, those in log phase, and after maximal physiologic stimulation (G0/G1). The DNAse I sites found at each of the time points (G0, 0.5, 1, 2, 3 hours after serum release and during log phase growth) are identical to those described previously for HFF (sites 1,2,3,4, and 6, figure 27). These data are consistent with those reported by Blanchard et al. (19) which suggest that cellular levels of c-myc transcript are primarily regulated by post-transcriptional mechanisms at the level of message degredation in normal cells.

C-myc chromatin structure analyses reported here, and those previously reported for lymphocytes provide important data as to the nature of regulatory interactions taking place with amplified c-myc in MFHs. First, chromatin structure data from quiescent and serum released HFF fibroblasts

indicate that in normal cells, post-transcriptional regulation is adequate to control **c-myc** transcript levels.

When the **c-myc** gene is amplified in MFHs, which are tumors of fibroblast origin, the normal post-transcriptional regulatory mechanisms may not be sufficient to compensate for the abundance of **c-myc** transcript produced. Changes in chromatin structure suggest that regulatory changes take place at the level of the gene as well. These changes in chromatin structure are indicative of a cellular adjustment to an abundance of **myc** expression. It is therefore concluded that the differences in regulation between amplified and single copy **c-myc** in MFHs may represent a compensatory response to gene dosage effects.

Western blot analysis showed that P3C cells have increased amounts of **c-myc** protein compared to UR HCL 1 cells or normal HFF fibroblasts 1 hour after serum release from G0. This suggests that **c-myc** gene amplification and increased transcript levels have an impact on the P3C cells through increased amounts of protein. These data further indicate that despite any attempt by the P3C cells to compensate for increased **c-myc** transcript, relatively high levels of **c-myc** protein are produced. This is further evidence that **c-myc** may be an oncogene in these cells.

Exactly what all of the consequences of **c-myc** gene amplfication in MFHs are, and whether multiple copies of the c-myc gene are the only abnormal events influencing increased levels of transcript production are questions yet to be answered. **C-myc** gene amplification may be a mechanism by tumor cells to obtain growth advantages over other surrounding cells. It would be interesting to study this *in vivo*. Individuals with neoplastic disease could be evaluated for various parameters of tumor growth. These include tumor stage, tumor size, angiogenesis factors, and metastasis. In order for a study of this nature to be meaningful, a large sample size and careful patient followups would be required. Although this would take years to complete, studies such as this would provide a more complete understanding of how abnormal gene copy number may actually effect tumor growth in individuals with cancer.

The following statements summarize the results from this project.

1. Increased transcript levels of **c-myc** and **c-sis** were observed in MFHs *in vivo*.
2. The c-sis gene is single copy in MFHs, therefore transcript levels are increased by an unknown mechanism other that gene amplification.

3. Increased **c-myc** transcript levels and TK:actin RNA ratios correlated with **c-myc** gene amplification *in vivo*.
4. Chromatin structure studies indicate that regulation of **c-myc** in normal fibroblasts is post-transcriptional.
5. When **c-myc** is amplified in MFH cells, changes in chromatin structure may represent a compensatory response to increased transcript levels.
6. Transcriptional and translational changes suggest that amplification of **c-myc** in P3C cells represents activation of a proto-oncogene to an oncogene.
7. **C-myc** gene amplification may be driving cell division in MFHs.
8. *In vitro* studies support the notion that **c-myc** gene amplfication may provide a selective growth advantage to MFH cells.

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BIOGRAPHICAL SKETCH

Jane Carolyn Strandberg Gibson was born in Madison, Wisconsin, on September 29, 1962, to James and Kathleen Strandberg, and is the oldest of their 3 children.

Jane has lived in the Orlando, Florida, area since 1969, where she graduated from Bishop Moore High School in 1980. During that same year, she began her college career at the University of Central Florida in Orlando. In 1982 she moved to Gainesville and attended the University of Florida were she received a Bachelor of Science degree in microbiology and cell science in April,1984.

In the fall of 1984, she began Graduate School as a student in the Department of Pathology at the University of Florida. In August 1986, she received her Master of Science degree in medical sciences-pathology. At that time she decided to continue her work with proto-oncogene expression in human sarcomas under the direction of Dr. Byron Croker. Jane married Ronald Lee Gibson on May 7, 1988. Since that time she has continued her work toward a Doctor of Philosophy degree in the Department of Pathology and Laboratory Medicine, University of Florida College of Medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1989

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